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(54) Luciferase gene and novel recombinant DNA as well as a method for production of luciferase.

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(73) Proprietor: KIKKOMAN CORPORATION
339 Noda
Noda-shi(JP)

(72) Inventor: Tatsumi, Hiroki
Kikkoman Apartment B-306
65-1, Yanagisawa Noda-shi(JP)
Inventor: Kajiyama, Naoki
101-2, Miyazaki
Noda-shi(JP)
Inventor: Nakano, Eiichi
2-86, Kizora
Iwatsuki-shi(JP)

(74) Representative: Kolb, Helga, Dr. Dipl.-Chem. et al
Hoffmann, Eitle & Partner,
Patentanwälte,
Postfach 81 04 20
D-81904 München (DE)

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Description

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a luciferase gene derived from Luciola lateralis (HEIKE firefly) and a novel recombinant DNA having integrated the gene therein as well as a method of producing a luciferase using the recombinant DNA.

2. Description of the Prior Art

Luciferase from fireflies belonging to the genus Luciola is merely obtained by isolating and purifying from the collected fireflies belonging to the genus Luciola [Proc. Natl. Acad. Sci., 74 (7), 2799-2802 (1977)].

Luciferases are very effectively usable, e.g., for quantitative determination of ATP.

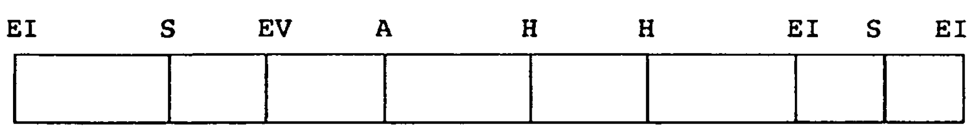
Since luciferases described above are derived from insects, however, fireflies belonging to the genus Luciola must be collected from the natural world to produce luciferases; alternatively, such fireflies must be cultivated and luciferases should be isolated and refined from the fireflies so that much time and labors are required for the production.

As a result of various investigations to solve the foregoing problems, the present inventors have found that by producing a recombinant DNA by inserting DNA containing a Luciola lateralis-derived luciferase-coding gene into a vector DNA and culturing in a medium a luciferase-producing microorganism belonging to the genus Escherichia and bearing the recombinant DNA, luciferase can be efficiently produced in a short period of time. As a result of further investigations on luciferase gene derived from Luciola lateralis, the present inventors have also succeeded in isolating a luciferase gene derived from Luciola lateralis and determining its structure, for the first time. This invention has thus been accomplished.

SUMMARY OF THE INVENTION

According to this invention, there are provided:

(1) A Luciola lateralis-derived luciferase gene defined by a restriction enzyme map described below:



wherein EI represents Eco RI, S represents Ssp I, EV represents Eco RV, A represents Apa I and H represents Hpa I.

(2) A luciferase gene according to (1) which encodes an amino acid sequence shown below:

```
                                                10
Met Glu Asn Met Glu Asn Asp Glu Asn Ile
                                                20
Val Tyr Gly Pro Glu Pro Phe Tyr Pro Ile
                                                30
Glu Glu Gly Ser Ala Gly Ala Gln Leu Arg
                                                40
Lys Tyr Met Asp Arg Tyr Ala Lys Leu Gly
                                                50
Ala Ile Ala Phe Thr Asn Ala Leu Thr Gly
                                                60
Val Asp Tyr Thr Tyr Ala Glu Tyr Leu Glu
                                                70
Lys Ser Cys Cys Leu Gly Glu Ala Leu Lys
                                                80
Asn Tyr Gly Leu Val Val Asp Gly Arg Ile
                                                90
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe
                                               100
Phe Ile Pro Val Leu Ala Gly Leu Phe Ile
                                               110
Gly Val Gly Val Ala Pro Thr Asn Glu Ile
                                               120
Tyr Thr Leu Arg Glu Leu Val His Ser Leu
                                               130
Gly Ile Ser Lys Pro Thr Ile Val Phe Ser
                                               140
Ser Lys Lys Gly Leu Asp Lys Val Ile Thr
                                               150
Val Gln Lys Thr Val Thr Ala Ile Lys Thr
                                               160
Ile Val Ile Leu Asp Ser Lys Val Asp Tyr
                                               170
Arg Gly Tyr Gln Ser Met Asp Asn Phe Ile
                                               180
Lys Lys Asn Thr Pro Gln Gly Phe Lys Gly
                                               190
Ser Ser Phe Lys Thr Val Glu Val Asn Arg
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									200
Lys	Glu	Gln	Val	Ala	Leu	Ile	Met	Asn	Ser
									210
Ser	Gly	Ser	Thr	Gly	Leu	Pro	Lys	Gly	Val
									220
Gln	Leu	Thr	His	Glu	Asn	Ala	Val	Thr	Arg
									230
Phe	Ser	His	Ala	Arg	Asp	Pro	Ile	Tyr	Gly
									240
Asn	Gln	Val	Ser	Pro	Gly	Thr	Ala	Ile	Leu
									250
Thr	Val	Val	Pro	Phe	His	His	Gly	Phe	Gly
									260
Met	Phe	Thr	Thr	Leu	Gly	Tyr	Leu	Thr	Cys
									270
Gly	Phe	Arg	Ile	Val	Met	Leu	Thr	Lys	Phe
									280
Asp	Glu	Glu	Thr	Phe	Leu	Lys	Thr	Leu	Gln
									290
Asp	Tyr	Lys	Cys	Ser	Ser	Val	Ile	Leu	Val
									300
Pro	Thr	Leu	Phe	Ala	Ile	Leu	Asn	Arg	Ser
									310
Glu	Leu	Leu	Asp	Lys	Tyr	Asp	Leu	Ser	Asn
									320
Leu	Val	Glu	Ile	Ala	Ser	Gly	Gly	Ala	Pro
									330
Leu	Ser	Lys	Glu	Ile	Gly	Glu	Ala	Val	Ala
									340
Arg	Arg	Phe	Asn	Leu	Pro	Gly	Val	Arg	Gln
									350
Gly	Tyr	Gly	Leu	Thr	Glu	Thr	Thr	Ser	Ala
									360
Ile	Ile	Ile	Thr	Pro	Glu	Gly	Asp	Asp	Lys
									370
Pro	Gly	Ala	Ser	Gly	Lys	Val	Val	Pro	Leu
									380
Phe	Lys	Ala	Lys	Val	Ile	Asp	Leu	Asp	Thr

390
Lys Lys Thr Leu Gly Pro Asn Arg Arg Gly
400
Glu Val Cys Val Lys Gly Pro Met Leu Met
410
Lys Gly Tyr Val Asp Asn Pro Glu Ala Thr
420
Arg Glu Ile Ile Asp Glu Glu Gly Trp Leu
430
His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu
440
Glu Lys His Phe Phe Ile Val Asp Arg Leu
450
Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
460
Val Pro Pro Ala Glu Leu Glu Ser Val Leu
470
Leu Gln His Pro Asn Ile Phe Asp Ala Gly
480
Val Ala Gly Val Pro Asp Pro Ile Ala Gly
490
Glu Leu Pro Gly Ala Val Val Val Leu Glu
500
Lys Gly Lys Ser Met Thr Glu Lys Glu Val
510
Met Asp Tyr Val Ala Ser Gln Val Ser Asn
520
Ala Lys Arg Leu Arg Gly Gly Val Arg Phe
530
Val Asp Glu Val Pro Lys Gly Leu Thr Gly
540
Lys Ile Asp Gly Lys Ala Ile Arg Glu Ile

Leu Lys Lys Pro Val Ala Lys Met

(3) A luciferase gene according to (1) or (2) which is represented by a nucleotide sequence shown below.

```
                                                30
ATG GAA AAC ATG GAG AAC GAT GAA AAT ATT
                                                60
GTG TAT GGT CCT GAA CCA TTT TAC CCT ATT
                                                90
GAA GAG GGA TCT GCT GGA GCA CAA TTG CGC
                                               120
AAG TAT ATG GAT CGA TAT GCA AAA CTT GGA
                                               150
GCA ATT GCT TTT ACT AAC GCA CTT ACC GGT
                                               180
GTC GAT TAT ACG TAC GCC GAA TAC TTA GAA
                                               210
AAA TCA TGC TGT CTA GGA GAG GCT TTA AAG
                                               240
AAT TAT GGT TTG GTT GTT GAT GGA AGA ATT
                                               270
GCG TTA TGC AGT GAA AAC TGT GAA GAA TTC
                                               300
TTT ATT CCT GTA TTA GCC GGT TTA TTT ATA
                                               330
GGT GTC GGT GTG GCT CCA ACT AAT GAG ATT
                                               360
TAC ACT CTA CGT GAA TTG GTT CAC AGT TTA
                                               390
GGC ATC TCT AAG CCA ACA ATT GTA TTT AGT
                                               420
TCT AAA AAA GGA TTA GAT AAA GTT ATA ACT
                                               450
GTA CAA AAA ACG GTA ACT GCT ATT AAA ACC
                                               480
ATT GTT ATA TTG GAC AGC AAA GTG GAT TAT
                                               510
AGA GGT TAT CAA TCC ATG GAC AAC TTT ATT
                                               540
AAA AAA AAC ACT CCA CAA GGT TTC AAA GGA
                                               570
TCA AGT TTT AAA ACT GTA GAA GTT AAC CGC
                                               600
AAA GAA CAA GTT GCT CTT ATA ATG AAC TCT
                                               630
TCG GGT TCA ACC GGT TTG CCA AAA GGT GTG
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                                              660
CAA CTT ACT CAT GAA AAT GCA GTC ACT AGA
                                              690
TTT TCT CAC GCT AGA GAT CCA ATT TAT GGA
                                              720
AAC CAA GTT TCA CCA GGC ACG GCT ATT TTA
                                              750
ACT GTA GTA CCA TTC CAT CAT GGT TTT GGT
                                              780
ATG TTT ACT ACT TTA GGC TAT CTA ACT TGT
                                              810
GGT TTT CGT ATT GTC ATG TTA ACG AAA TTT
                                              840
GAC GAA GAG ACT TTT TTA AAA ACA CTG CAA
                                              870
GAT TAC AAA TGT TCA AGC GTT ATT CTT GTA
                                              900
CCG ACT TTG TTT GCA ATT CTT AAT AGA AGT
                                              930
GAA TTA CTC GAT AAA TAT GAT TTA TCA AAT
                                              960
TTA GTT GAA ATT GCA TCT GGC GGA GCA CCT
                                              990
TTA TCT AAA GAA ATT GGT GAA GCT GTT GCT
                                             1020
AGA CGT TTT AAT TTA CCG GGT GTT CGT CAA
                                             1050
GGC TAT GGT TTA ACA GAA ACA ACC TCT GCA
                                             1080
ATT ATT ATC ACA CCG GAA GGC GAT GAT AAA
                                             1110
CCA GGT GCT TCT GGC AAA GTT GTG CCA TTA
                                             1140
TTT AAA GCA AAA GTT ATC GAT CTT GAT ACT
                                             1170
AAA AAA ACT TTG GGC CCG AAC AGA CGT GGA
                                             1200
GAA GTT TGT GTA AAG GGT CCT ATG CTT ATG
                                             1230
AAA GGT TAT GTA GAT AAT CCA GAA GCA ACA
                                             1260
AGA GAA ATC ATA GAT GAA GAA GGT TGG TTG
                                             1290
CAC ACA GGA GAT ATT GGG TAT TAC GAT GAA
```

```
                                               1320
GAA AAA CAT TTC TTT ATC GTG GAT CGT TTG
                                               1350
AAG TCT TTA ATC AAA TAC AAA GGA TAT CAA
                                               1380
GTA CCA CCT GCT GAA TTA GAA TCT GTT CTT
                                               1410
TTG CAA CAT CCA AAT ATT TTT GAT GCC GGC
                                               1440
GTT GCT GGC GTT CCA GAT CCT ATA GCT GGT
                                               1470
GAG CTT CCG GGA GCT GTT GTT GTA CTT GAA
                                               1500
AAA GGA AAA TCT ATG ACT GAA AAA GAA GTA
                                               1530
ATG GAT TAC GTT GCT AGT CAA GTT TCA AAT
                                               1560
GCA AAA CGT TTG CGT GGT GGT GTC CGT TTT
                                               1590
GTG GAC GAA GTA CCT AAA GGT CTC ACT GGT
                                               1620
AAA ATT GAC GGT AAA GCA ATT AGA GAA ATA

CTG AAG AAA CCA GTT GCT AAG ATG
```

According to this invention, there is also provided a novel recombinant DNA obtained by inserting a gene coding for luciferase derived from Luciola lateralis into a vector DNA. According to this invention, there is further provided a method of producing a luciferase which comprises culturing in a medium a microorganism belonging to the genus Escherichia and bearing a recombinant DNA obtained by inserting a gene coding for luciferase derived from Luciola lateralis in a vector DNA, and collecting a luciferase from the culture.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an optimum pH range of luciferase derived from Luciola lateralis. Fig. 2 shows a stable pH range of luciferase derived from Luciola lateralis. Fig. 3 shows a cleavage map of recombinant plasmid pALf3 DNA with restriction enzymes. Fig. 4 shows a cleavage map of recombinant plasmid pGLf1 DNA with restriction enzymes. Fig. 5 shows a cleavage map of recombinant plasmid pHLf7 DNA with restriction enzymes. Fig. 6 shows a nucleotide sequence of Luciola lateralis-derived luciferase gene in accordance with this invention. Fig. 7 shows an amino acid sequence of polypeptide translated from Luciola lateralis-derived luciferase gene of this invention. Fig. 8 shows a nucleotide sequence of Luciola lateralis-derived luciferase gene and the amino acid sequence corresponding thereto.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Hereafter this invention is described in detail.

In survey of DNA bearing a gene encoding luciferase derived from Luciola lateralis (HEIKE firefly), DNA containing a gene encoding luciferase derived from Luciola cruciata (GENJI firefly) belonging to the same genus, which is one of fireflies, is used as a probe; further in survey of DNA bearing a Luciola cruciata-derived luciferase-coding gene, DNA containing a gene coding for luciferase derived from Photinus pyralis - (American firefly), which is one of fireflies, is used as a probe.

Therefore, production of the gene encoding luciferase from Photinus pyralis is firstly described below. Subsequently, production of the gene encoding luciferase from Luciola cruciata is described and finally, production of the gene encoding luciferase from Luciola lateralis is described.

To prepare m-RNA from the posterior portion of Photinus pyralis which is one of fireflies, m-RNA can be obtained according to methods described in, for example, Molecular Cloning, page 196, Cold Spring Harbor Laboratory (1982), Haruo Ozeki and Reiro Shimura, BUNSHI IDENGAKU JIKKENHO (Experimental Molecular Genetics), pages 66-67 (1983), etc.

Concentration of m-RNA coding for luciferase from the obtained m-RNA can be performed by a method described in, for example, Biomedical Research, 3, 534-540 (1982) or the like.

In this case, anti-luciferase serum to luciferase is used. This serum can be obtained by, for example, Yuichi Yamamura, MEN-EKI KAGAKU (Immunochemistry), pages 43-50 (1973), etc.

Synthesis of c-DNA from the m-RNA coding for luciferase can be performed by methods described in, for example, Mol. Cell Biol., 2, 161 (1982) and Gene, 25, 263 (1983).

Then, the thus obtained c-DNA is integrated into, for example, plasmid pMCE10 DNA {plasmid produced using plasmid pKN305 [plasmid having a promoter of Escherichia coli tryptophan operator described in Agr. Biol. Chem., 50, 271 (1986)] and plasmid pMC1843 [plasmid containing Escherichia coli β-galactosidase structural gene described in Methods in Enzymology, 100, 293-308 (1983)]}, etc. to produce various recombinant plasmid DNAs. Using these DNAs, transformation of Escherichia coli (E. coli) DH1 (ATCC 33849), E. coli HB101 (ATCC 33694), etc. is effected by the method of Hanahan [DNA Cloning, 1, 109-135 (1985)] to obtain various transformants.

The recombinant plasmid c-DNAs possessed by the thus obtained transformants are plasmids wherein c-DNA has been integrated in the middle of E. coli β-galactosidase structural gene. A peptide encoded by c-DNA is expressed as a protein fused with β-galactosidase.

In order to detect c-DNA coding for luciferase from the various transformants described above, the transformants are cultured thereby to express cell protein. By determining if any protein crossing over anti-luciferase serum is present, its detection can be made. Methods described in, for example, Agric. Biol. Chem., 50, 271 (1986) and Anal. Biochem., 112, 195 (1981), etc. can be used for the detection.

Next, after labeling c-DNA of incomplete luciferase with ^{32}P by the nick translation method [Molecular Cloning, pages 109-112, Cold spring Harbor Laboratory (1982) and J. Mol. Biol., 113, 237-251 (1977)], using the colony hybridization method [Protein, Nucleic Acid & Enzyme, 26, 575-579 (1981)], an Escherichia coli strain having plasmid DNA containing Photinus pyralis luciferase c-DNA of 1.8 Kb can be obtained from a Photinus pyralis-derived c-DNA library prepared using plasmid pUC19 DNA (manufactured by Takara Shuzo Co., Ltd.) as a vector.

To obtain a DNA containing the gene coding for luciferase derived from Photinus pyralis from the thus obtained recombinant plasmid DNA, restriction enzymes, e.g., Eco RI and Cla I, are acted on the plasmid DNA at temperatures of 30 to 40°C, preferably at 37°C, for 1 to 24 hours, preferably for 2 hours; the solution obtained after completion of the reaction is subjected to agarose gel electrophoresis [which is described in Molecular Cloning, page 150, Cold Spring Harbor Laboratory (1982)] to obtain the DNA containing the gene coding for luciferase derived from Photinus pyralis.

Next, production of the Luciola cruciata-derived luciferase-coding gene are described below.

Preparation of m-RNA from the posterior portion of Luciola cruciata and synthesis of c-DNA from the m-RNA can be conducted, for example, in quite the same manner as in the preparation of the m-RNA of Photinus pyralis and synthesis of the c-DNA described above.

Then, the thus obtained c-DNA is integrated into a vector DNA, for example, plasmid pUC19 DNA (manufactured by Takara Shuzo Co., Ltd.), etc. to obtain various recombinant plasmid DNAs. Using these DNAs, transformation of E. coli DH1 (ATCC 33849), E. coli HB101 (ATCC 33694), etc. is effected by the method of Hanahan [DNA Cloning, 1, 109-135 (1985)] to obtain various transformants.

Next, after labeling c-DNA of luciferase derived from Photinus pyralis with ^{32}P by the nick translation method [Molecular Cloning, pages 109-112, Cold Spring Harbor Laboratory (1982) and J. Mol. Biol., 113, 237-251 (1971)], using the colony hybridization method [Protein, Nucleic Acid & Enzyme, 26, 575-579 (1981)], an Escherichia coli strain having plasmid DNA containing Luciola cruciata luciferase c-DNA of 2.0 Kb can be obtained from a Luciola cruciata-derived c-DNA library prepared using plasmid pUC19 DNA (manufactured by Takara Shuzo Co., Ltd.) as a vector.

To obtain the purified plasmid DNA, there is used, for example, a method described in Proc. Natl. Acad. Sci., 62 1159-1166 (1969), etc.

By acting on the purified plasmid DNA, for example, restriction enzyme, e.g., Pst I (manufactured by Takara Shuzo Co., Ltd.) at a temperature of 30°C to 40°C, preferably at 37°C for 1 to 24 hours, preferably for 2 hours, the resulting solution obtained after completion of the reaction is subjected to agarose gel

electrophoresis [which is described in Molecular Cloning, page 150, Cold Spring Harbor Laboratory (1982)] to obtain the DNA containing the gene coding for luciferase derived from Luciola cruciata.

Next, production of the Luciola lateralis-derived luciferase-coding gene in accordance with this invention are described below.

Firstly, as source from which the m-RNA coding for luciferase derived from Luciola lateralis is collected, the posterior portion of Luciola lateralis is preferred since the m-RNA is present in the posterior portion of this firefly.

Preparation of m-RNA from the posterior portion of the firefly and synthesis of c-DNA from the m-RNA can be conducted, for example, in quite the same manner as in the preparation of the m-RNA of Photinus pyralis and synthesis of the c-DNA described above.

Then, the thus obtained c-DNA is integrated into a vector DNA, for example, plasmid pUC119 DNA, etc. to obtain various recombinant plasmid DNAs. Using these DNAs, transformation of E. coli DH1 (ATCC 33849), E. coli HB101 (ATCC 33694), etc. is effected by the method of Hanahan [DNA Cloning, 1, 109-135 (1985)] to obtain various transformants.

Next, after labeling c-DNA of luciferase derived from Luciola cruciata with ^{32}P by the nick translation method [Molecular Cloning, pages 109-112, Cold Spring Harbor Laboratory (1982) and J. Mol. Biol., 113, 237-251 (1977)], using the colony hybridization method [Protein, Nucleic Acid & Enzyme, 26, 575-579 (1981)], an Escherichia coli stran having plasmid DNA containing Luciola lateralis luciferase c-DNA of 2.0 Kb can be obtained from a Luciola lateralis-derived c-DNA library prepared using plasmid pUC119 DNA (manufactured by Takara Shuzo Co., Ltd.) as a vector.

To obtain the purified recombinant DNA from the thus obtained microorganism, there is used, for example, a method described in Proc. Natl. Acad. Sci., 62 1159-1166 (1969), etc.

By acting on the purified, new recombinant DNA, for example, 2 units of restriction enzyme Eco RI (manufactured by Takara Shuzo Co., Ltd.) at a temperature of 30°C or higher, preferably at 37°C, for 1 to 4 hours, preferably for 2 hours, partial digestion is effected. Then, digestion product is subjected to agarose gel electrophoresis to obtain 2,000 bp DNA fragment containing all the gene coding for luciferase derived from Luciola lateralis.

On the other hand, a nucleotide sequence of this luciferase gene is determined by the method as shown in Item 18 in the example. The determined nucleotide sequence is shown in Fig. 6. Subsequently, an amino acid sequence of polypeptide translated from the nucleotide sequence is identified. The results are shown in Fig. 7.

The gene encoding the thus identified amino acid sequence is also included in this invention.

The above-mentioned microorgansim is then cultured in a medium and luciferase is collected from the culture.

Any medium may be used as far as it is used to culture microorganisms belonging to the genus Escherichia. Mention may be made of, for example, 1% (W/V) of trypton, 0.5% (W/V) of yeast extract, 0.5% (W/V) of NaCl and 1 mM of isopropyl-β-D-thiogalactoside, etc.

Temperature for the cultivation is between 30 and 40°C, preferably about 37°C and a time period for the cultivation is, for example, 4 to 8 hours, preferably about 4 hours.

The cells are collected from the culture by centrifugation at 8,000 r.p.m. for about 10 minutes. The obtained cells are homogenized by the method described in, for example, Methods in Enzymology, 133, 3-14 (1986) to obtain a crude enzyme solution.

The crude enzyme solution may be usable as it is; if necessary and desired, the crude enzyme solution can be purified by fractionation with ammonium sulfate, hydrophobic chromatography (for example, using BUTYL TOYOPEARL 650C, etc.), gel filtration (using, e.g., Ultrogel AcA34, etc.) thereby to give purified luciferase.

Physicochemical properties of the thus obtained luciferase are as described below.

(1) Action

The enzyme catalyzes the oxidation of luciferin by an oxygen molecule, as shown by the enzymatic reaction equation:

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow{\text{Present enzyme}} \text{Oxyluciferin} + \text{AMP} + \text{Pyrophosphoric acid} + O_2 + \text{light}$$

(2) Substrate specificity

The enzyme does not act on ADP, CTP, UTP and GTP.

(3) Optimum pH, and pH range for stability

The optimum pH is, as shown in Fig. 1, 7.5 to 9.5 as measured by carrying out the reaction by the use of luciferin as a substrate at various pH values of 25 mM glycylglycine buffer solution in the range of 6.5 to 11.5 and at a temperature of 30 • C, and measuring the quantity of light (the number of photons) emitted in 20 seconds. The stable pH range of the enzyme is, as shown in Fig. 2, 6.0 to 10.5 as measured by adding the enzyme to each of buffer solutions [25 mM phosphate buffer solution (pH 4.6-8.0) and 25 mM glycine.sodium chloride-sodium hydroxide buffer solutions (pH 8.0 - 11.5), each of which contains ammonium sulfate to 10% saturation] containing luciferin, and allowing the enzyme to act at a temperature of 0 • C for 4 hours. In Fig. 2, O—O and Δ—Δ show the activity in the case of using the 25 mM phosphate buffer solutions and the activity in the case of using 25 mM glycine.sodium chloride-sodium hydroxide buffer solutions, respectively.

(4) Measurement of titer

A luciferin mixed solution is prepared by mixing 8 ml of 25 mM glycylglycine buffer solution (pH 7.8), 0.5 ml of a magnesium sulfate solution [a solution prepared by adding magnesium sulfate to 25 mM glycylglycine buffer solution (pH 7.8), a magnesium sulfate concentration of 0.1 M] and 0.8 ml of a luciferin solution [a solution prepared adding luciferin to 25 mM glycylglycine buffer solution (pH 7.8), a luciferin concentration of 1 mM].

Into a mixture of 400 µl of the luciferin mixed solution thus obtained and 10 µl of luciferase to be assayed is poured 80 µl of an ATP solution [a solution prepared by adding ATP to 25 mM glycylglycine buffer solution (pH 7.8), an ATP concentration of 10 mM]. Simultaneously with the pouring, the number of photons generated is measured by adding up for 20 seconds by means of a luminometer (LUMINESCENCE READER BLR-201, manufactured by ALOKA Co., Ltd.).

(5) Ragne of temperature suitable for action

When the reaction is carried out at pH 7.8 at each temperature and the quantity of light (the number of photons) emitted in 20 seconds is measured, the suitable temperature for the action ranges from 0 • to 50 • C.

(6) Conditions for inactivation by pH

At pH values of 5.0 or lower and 12.0 or higher, the enzyme is completely inactivated 4 hours after.

As is clear from the foregoing description, according to this invention, luciferase can be efficiently produced in an extremely short period of time, by culturing the microorgnism belonging to the genus Escherichia which contains the recombinant DNA having integrated therein the Luciola lateralis-derived luciferase gene of this invention. Therefore, this invention is extremely useful from an industrial point of view.

Hereafter this invention will be described in more detail by referring to the examples below.

Example

In Items 1 to 10 below, production of DNA containing a gene coding for luciferase of Photinus pyralis as one of fireflies (this DNA is used as a probe upon survey of DNA containing a gene coding for luciferase of Luciola cruciata) is described. Further in Items 11 to 13 below, production of DNA containing a gene coding for luciferase derived from Luciola cruciata (this DNA is used as a probe upon survey of DNA containing a gene coding for luciferase of Luciola lateralis) is described.

1. Preparation of m-RNA

Using a mortar and a pestle, 1 g of the dry posterior portion (manufactured by Sigma Co., Ltd.) of Photinus pyralis as one of fireflies was thoroughly ground, to which 5 ml of dissolution buffer [20 mM Tris-hydrochloride buffer (pH 7.4)/10 mM NaCl/3 mM magnesium acetate/5% (W/V) sucrose/1.2 % (V/V) Triton X-100/10 mM vanadyl nucleoside complex (manufactured by New England Biolab Co., Ltd.)] was added. The mixture was further ground as in the manner described above to give a solution containing the ground posterior portion of Photinus pyralis.

In a cup blender (manufactured by Nippon Seiki Seisakusho) was charged 5 ml of the thus obtained solution. After treating at 5,000 r.p.m. for 5 minutes, 12 ml of guanidine isothiocyanate solution (6M guanidine isothiocyanate/37.5 mM sodium citrate (pH 7.0)/0.75% (W/V) sodium N-lauroylsarcocine/0.15 M β-mercaptoethanol) was added to the system. The mixture was treated with the blender described above at 3,000 r.p.m. for 10 minutes. The resulting solution was filtered using a threefold gauze to give the filtrate. The filtrate was gently poured in layers onto 4 tubes for ultracentrifuging machine (manufactured by Hitachi Koki Co., Ltd.) in which 1.2 ml each of 5.7 M cesium chloride solution had previously be laid in layers. Using the ultracentrifuging machine (manufactured by Hitachi Koki Co., Ltd., SCP55H), centrifugation was performed at 30,000 r.p.m. for 16 hours to give precipitates.

The obtained precipitates were washed with cold 70% (V/V) ethanol and suspended in 4 ml of 10 mM Tris buffer [10 mM Tris-hydrochloride (pH 7.4)/5 mM EDTA/1% sodium dodecylsulfate]. The equal amount of a mixture of n-butanol and chloroform in 1 : 4 (volume ratio) was added to the mixture to perform extraction. The extract was centrifuged at 3,000 r.p.m. for 10 minutes in a conventional manner to separate into the aqueous phase and the organic solvent phase. To the organic solvent phase was added 4 ml of 10 mM Tris buffer described above. The procedure for the extraction and separation described above was repeated twice. To the aqueous phase obtained were added a 1/10 amount of 3 M sodium acetate (pH 5.2) and a 2-fold amount of cold ethanol were added. After allowing to stand at a temperature of -20 °C for 2 hours, the mixture was centrifuged at 8,000 r.p.m. for 20 minutes in a conventional manner to precipitate RNA. The obtained RNA was dissolved in 4 ml of water. After the operation for precipitation with ethanol described above was carried out, the obtained RNA was dissolved in 1 ml of water to give 3.75 mg of RNA.

By repeating the foregoing procedure again, 7 mg in total of RNA was prepared. To select m-RNA from the RNA, 7 mg of RNA was subjected to oligo(dT)-cellulose (manufactured by New England Biolab Co., Ltd.) column chromatography.

As the column, 2.5 ml of Terumo syringe (manufactured by Terumo Co., Ltd.) was used. After 0.5 g of resin was swollen with elution buffer [10 mM Tris-hydrochloride buffer (pH 7.6)/1 mM DETA/0.1% (W/V) sodium dodecylsulfate], the resin was packed in the column and equilibrated with binding buffer [10 mM Tris-hydrochloride (pH 7.6)/1 mM EDTA/0.4 M NaCl/0.1% (W/V) sodium dodecylsulfate].

To 7 mg of RNA was added the same amount of buffer [10 mM Tris-hydrochloride (pH 7.6)/1 mM EDTA/0.8 M NaCl/0.1% (W/V) sodium dodecylsulfate]. The mixture was heat-treated at a temperature of 65 °C for 10 minutes and then quenched in ice water. After subjecting to oligo(dT)-celluose column, the resin was washed with binding buffer to completely wash unbound r-RNA and t-RNA out. Further m-RNA was eluted with eluting buffer to give 40 μg of m-RNA.

2. Concentration of luciferase m-RNA

Next, the luciferase m-RNA was concentrated by sucrose density gradient centrifugation.

Sucrose density gradient of 10 to 25% (W/V) was produced by charging 0.5 ml of 40% (W/V) sucrose solution [50 mM Tris-hydrochloride buffer (pH 7.5)/20 mM NaCl/1 mM EDTA/40% (W/V) sucrose] in a polyaroma tube for Rotor SW41 manufactured by Beckmann Co., Ltd., laying 2.4 ml each of 25% (W/V), 20% (W/V), 15% (W/V) and 10% (W/V) of the sucrose solution in layers and allowing to stand the system at a temperature of 4 °C for 24 hours. To the sucrose density gradient, 30 μg of m-RNA was laid to form a layer. Using SW41 Rotor manufactured by Beckmann Co., Ltd., centrifugation was conducted at 30,000

r.p.m. at a temperature of 18°C for 18 hours in a conventional manner. After the centrifuging operation, fractionation was performed by 0.5 ml each and m-RNA was recovered by the ethanol precipitation method. The m-RNA was dissolved in 10 µl of water.

Next, protein encoded by the m-RNA was examined, whereby the fraction concentrated on m-RNA of luciferase was identified. One microliter of the fractionated RNA, 9 µl of rabbit reticular erythrocyte lysate (manufactured by Amersham Co., Ltd.) and 1 µl of [^{35}S] methionine (manufactured by Amersham Co., Ltd.) were mixed and reacted at a temperature of 30°C for 30 minutes. To the reaction mixture was added 150 µl of NET buffer [150 mM NaCl/5 mM EDTA/0.02% (W/V) NaN_3/20 mM Tris-hydrochloride buffer (pH 7.4)-/0.05% (W/V) Nonidet P-40 (manufactured by Besesda Research Laboratories Co., Ltd., surface active agent)] and, 1 µl of antiluciferase serum (produced as will be later described) was added to the mixture. After allowing to stand at a temperature of 20°C for 30 hours, 10 mg of Protein A Sepharose (manufactured by Pharmacia Fine Chemicals Inc.) was added to the mixture. The resulting mixture was then centrifuged at 12,000 r.p.m. for a minute in a conventional manner to recover the resin.

The recovered resin was washed three times with 200 µl of NET buffer. To the resin was added 40 µl of sample buffer for SDS-PAGE [62.5 mM Tris-hydrochloride buffer (pH 6.8)/10% (V/V) glycerol/2% (W/V) sodium dodecylsulfate/5% (V/V) β-mercaptoethanol/0.02% (W/V) bromophenol blue]. The mixture was boiled at a temperature of 100°C for 3 minutes and centrifuged at 12,000 r.p.m. for a minute in a conventional manner to recover the supernatant. The whole amount was applied onto 7.5% (W/V) sodium dodecylsulfate-polyacrylamide gel.

Gel electrophoresis was performed by the method of Laemmli [Nature, 227, 680 (1970)]. After the electrophoresis, the gel was immersed in 10% (V/V) acetic acid for 30 minutes to immobilize protein. Then, the gel was immersed in water for 30 minutes and further immersed in 1 M sodium salicylate solution for 30 minutes and then dried to give a dry gel. The dry gel was subjected to fluorography using an X ray film (manufactured by Fuji Photo Film Co., Ltd.; RX).

By the foregoing procedure, the band of luciferase protein was recognized on the X ray film only in the case of using the RNA from the fraction in which the luciferase m-RNA was present and, the fraction wherein the luciferase m-RNA was concentrated could be identified.

3. Production of anti-serum

Rabbit anti-luciferase serum to purified luciferase was produced by the following method.

A luciferase solution having a 3.2 mg/ml concentration [solution obtained by dissolving luciferase manufactured by Sigma Co., Ltd. in 0.5 M glycylglycine solution (pH 7.8)], 0.7 ml, was suspended in an equal amount of Freund's complete adjuvant. 2.24 mg of the suspension was administered as an antigen to the palm of Japanese white rabbit weighing 2 kg as an antigen. After feeding for 2 weeks, the same amount of antigen as in the initial amount was intracutaneously administered to the back. After feeding for further one week, similar procedure was performed. Further one week after feeding, whole blood was collected.

The obtained blood was allowed to stand at a temperature of 4°C for 18 hours and then centrifuged at 3,000 r.p.m. for 15 minutes in a conventional manner to give anti-luciferase serum as the supernatant.

4. Synthesis of c-DNA

Synthesis of c-DNA was carried out using a kit manufactured by Amersham Co., Ltd.

Using 2 µg of m-RNA obtained as described above, synthesis of c-DNA was carried out in accordance with the methods described in Mol. Cell Biol., 2, 161 (1982) and Gene, 25, 263 (1983). As the result, 300 ng of double stranded c-DNA was obtained.

This c-DNA, 150 ng, was dissolved in 7 µl of TE buffer [10 mM Tris-hydrochloride buffer (pH 7.5)/1 mM EDTA]. To the solution were added, respectively, 11 µl of a mixture [280 mM sodium cacodylate (pH 6.8)-/60 mM Tris-hydrochloride buffer (pH 6.8)/2 mM cobalt chloride] and 3.8 µl of a tailing mixture [7.5 µl of 10 mM dithiothreitol/1 µl of 10 ng/ml poly(A)/2 µl of 5mM dCTP/110 µl of water]. Furthermore, 29 units of terminal transferase (manufactured by Boehringer Mannheim GmbH) was added to the mixture. After reacting at a temperature of 30°C for 10 minutes, 2.4 µl of 0.25 M EDTA and 2.4 µl of 10% (W/V) sodium dodecylsulfate were added to the mixture to discontinue the reaction.

The solution in which the reaction had been discontinued was subjected to a treatment for removing protein using 25 µl of water-saturated phenol. Then, 25 µl of 4 M ammonium acetate and 100 µl of cold ethanol were added to the recovered aqueous phase, respectively. The mixture was allowed to stand at a temperature of - 70°C for. 15 minutes and centrifuged at 12,000 r.p.m. for 10 minutes to recover c-DNA. The c-DNA was dissolved in 10 µl of TE buffer to give a c-DNA solution.

As described above, 100 ng of the c-DNA with the deoxycytidine tail was obtained.

5. Production of recombinant plasmid pMCE10 DNA used in vector

Plasmid pKN305 DNA produced by the method described in T. Masuda et al., Agricultural Biological Chemistry, 50, 271-279 (1986) using plasmid pBR325 (manufactured by BRL Co.) and plasmid pBR322 DNA (manufactured by Takara Shuzo Co., Ltd.), and pMC1403-3 DNA (described in Japanese Patent Publication KOKAI 61-274683) were added by 1 µg each to 10 µl of a mixture [50 mM Tris-hydrochloride buffer (pH 7.5)/10 mM $MgCl_2$/100 mM NaCl/1 mM dithiothreitol]. Further, 2 units each of Hind III and Sal I (both manufactured by Takara Shuzo Co., Ltd.) were added to the mixture. By reacting at a temperature of 37°C for an hour, a cleavage treatment was effected. Extraction with phenol and precipitation with ethanol were conducted in a conventional manner to give precipitates. The precipitates were dissolved in 10 µl of ligation buffer [20 mM $MgCl_2$/66 mM Tris-hydrochloride buffer (pH 7.6)/1 mM ATP/15 mM dithiothreitol] to give a solution. Furthermore, 1 unit of T4 DNA ligase (manufactured by Takara Shuzo Co., Ltd.) was added thereto to perform ligation at a temperature of 20°C for 4 hours. Then, using this reaction solution, E. coli JM101 (ATCC 33876) was transformed according to the transformation method described in [J. Bacteriology, 119, 1072-1074 (1974)]. By examination of chemical resistance (ampicillin resistance and tetracycline sensitivity) and β-galactosidase activity, a transformant was obtained. Recombinant plasmid DNA contained in the strain was named pMCE10. E. coli JM101 strain containing this recombinant plasmid DNA pMCE10 DNA was cultured in medium composed of 1% (W/V) of trypton, 0.5% (W/V) of yeast extract and 0.5% (W/V) of NaCl at a temperature of 37°C for 16 to 24 hours. Twenty milliliters of the thus obtained culture solution of E. coli JM101 (pMCE10) was inoculated on 1 liter of the medium followed by shake culture at a temperature of 37°C for 3 hours. After the addition of 0.2 g of chloramphenicol, cultivation was conducted at the same temperature for further 20 hours to give a culture solution.

Next, the culture solution was centrifuged at 6,000 r.p.m. for 10 minutes in a conventional manner to give 2 g of wet cells. After the cells were suspended in 20 ml of 350 mM Tris-hydrochloride buffer (pH 8.0) containing 25% (W/V) sucrose, 10 mg of lysozyme, 8 ml of 0.25 M EDTA solution (pH 8.0) and 8 ml of 20% (W/V) sodium dodecylsulfate were added to the suspension, respectively. The mixture was kept at a temperature of 60°C for 30 minutes to give a lysate solution.

To the lysate solution was added 13 ml of 5 M NaCl solution. The mixture was treated at a temperature of 4°C for 16 hours and then centrifuged at 15,000 r.p.m. in a conventional manner to give an extract. The extract was subjected to the phenol extraction and the ethanol precipitation in a conventional manner to give precipitates.

Then, the precipitates were dried under reduced pressure in a conventional manner and dissolved in 10 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM EDTA. To the solution were further added 6 g of cesium chloride and 0.2 ml of ethydium bromide solution (10 mg/ml). The resulting mixture was subjected to an equilibrated density gradinent centrifugation treatment using a ultracentrifuging machine at 39,000 r.p.m. for 42 hours in a conventional manner thereby to isolate recombinant plasmid pMCE10 DNA. After ethydium bromide was removed using n-butanol, dialysis was performed to 10 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM EDTA to give 500 µg of purified recombinant plasmid pMCE10 DNA.

6. Production of vector DNA

The thus obtained recombinant plasmid pMCE10 DNA, 15 µg, was dissolved in 90 µl of TE buffer described in Item 4. After 10 µl of Med buffer [100 mM Tris-hydrochloride buffer (pH 7.5)/10 mM $MgCl_2$/10 mM dithiothreitol/500 mM NaCl] was added to the solution, 30 units of restriction enzyme Acc I (manufactured by Takara Shuzo Co., Ltd.) was further added to the mixture. A cleavage treatment was conducted at a temperature of 37°C for an hour to give the cleavage product. To the cleavage product was added 100 µl of water-saturated phenol, whereby protein was removed. Then, the aqueous phase was recovered and a 1/10-fold amount of 3 M sodium acetate (pH 7.5) and a 2-fold amount of cold ethanol were added to the aqueous phase. After allowing to stand at a temperature of -70°C for 15 minutes, the mixture was centrifuged at 12,000 r.p.m. for 10 minutes to recover DNA.

This DNA was dissolved in 10 µl of TE buffer and 15 µl of a mixture [280 mM sodium cacodylate (pH 6.8)/60 mM Tris-hydrochloride buffer (pH 6.8)/2 mM cobalt chloride] was added to the solution. Then, 5 µl of a tailing solution mixture (described in Item 4) (5 mM dGTP was used) was further added to the mixture. Furthermore, 5 units of terminal transferase (manufactured by Takara Shuzo Co., Ltd.) was added to react at a temperature of 37°C for 15 minutes. By post-treatment in a manner similar to the c-DNA tailing reaction described in Item 4, DNA with the deoxyguanosine tail at the Acc I site of recombinant plasmid pMCE10

DNA was produced.

On the other hand, DNA with the deoxyguanosine tail at the Pst I site of plasmid pUC19 DNA was also produced at the same time.

To a solution of 30 µg of plasmid pUC19 DNA (manufactured by Takara Shuzo Co., Ltd.) in 350 µl of TE buffer were added 40 µl of Med buffer and 120 units of restriction enzyme Pst I (manufactured by Takara Shuzo Co., Ltd.). After a cleavage treatment at a temperature of 37 °C for an hour, DNA was recovered by the phenol treatment for removing protein and ethanol precipitation in a conventional manner.

The obtained DNA was dissolved in 35 µl of TE buffer. To the solution were added 50 µl of a mixture [280 mM sodium cacodylate (pH 6.8)/60 mM Tris-hydrochloride buffer (pH 6.8)/1 mM cobalt chloride], 19 µl of the tailing mixture (containing dGTP instead of dCTP) described in Item 4 and 60 units of terminal transferase (manufactured by Takara Shuzo Co., Ltd.). After reacting at a temperature of 37 °C for 10 minutes, DNA was recovered by the phenol treatment for removing protein and ethanol precipitation in a conventional manner.

7. Annealing and transformation

The thus synthesized c-DNA, 15 ng and 200 ng of vector DNA were dissolved in 35 µl of annealing buffer [10 mM Tris-hydrochloride buffer (pH 7.5)/100 mM NaCl/1 mM EDTA]. The solution was allowed to stand at a temperature of 65 °C for 2 minutes, at a temperature of 46 °C for 2 hours, at a temperature of 37 °C for an hour and at a temperature of 20 °C for 18 hours thereby to anneal c-DNA and vector DNA.

Using the annealed DNA, E. coli DH1 strain (ATCC 33849) was transformed by the method of Hanahan [DNA Cloning, 1, 109-135 (1985)] to produce a c-DNA bank containing plasmid pUC19 DNA and recombinant plasmid pMCE10 DNA as vectors, respectively.

8. Survey of luciferase c-DNA

The Acc I site of recombinant plasmid pMCE10 DNA is present at a site which codes for E. coli B-galactosidase gene. Therefore, c-DNA incorporated into this site forms a fused protein with β-galactosidase. Furthermore, a promoter of β-galactosidase gene of the recombinant plasmid pMCE10 DNA has been converted into a promoter of E. coli tryptophan gene, as described above.

96 colonies of c-DNA having recombinant plasmid pMCE10 DNA as a vector were shake cultured in 10 ml of M9 Casamino acid medium [Molecular Cloning, 440-441, Cold Spring Harbor Laboratory (1982)] supplemented with thiamine (10 µg/ml) at a temperature of 37 °C for 10 hours. After collecting the cells in a conventional manner, the cells were suspended in 200 µl of sample buffer for SDS-PAGE described in Item 2. The suspension was boiled at a temperature of 100 °C for 5 minutes. This suspension, 40 µl, was subjected to electrophoresis in a conventional manner using 7.5% (W/V) polyacrylamide gel. After completion of the electrophoresis, the protein developed on the gel was transferred onto a nitrocellulose filter by the western blot method [Anal. Biochem., 112, 195 (1981)]. This nitrocellulose filter was stained with anti-luciferase serum using immune blot assay kit (manufactured by Biorad Co.). The method was performed in accordance with the instruction of Biorad Co.

That is, the nitrocellulose filter was shaken in 100 ml of blocking solution [a solution obtained by dissolving 3% (W/V) gelatin in TBS buffer [20 mM Tris-hydrochloride buffer /500 mM NaCl (pH 7.5)] at a temperature of 25 °C for 30 minutes. Next, this nitrocellulose filter was transferred into 25 ml of primary antibody solution [solution obtained by dissolving 1% (W/V) gelatin in TBS buffer and diluting luciferase anti-serum with the resulting solution] and shaken at a temperature of 25 °C for 90 minutes, which was then transferred into 100 ml Tween-20 washing solution [solution obtained by dissolving 0.05% (W/V) Tween-20 in TBS buffer] and shaken at a temperature of 25 °C for 10 minutes. This procedure was repeated twice. Then, the thus obtained nitrocellulose filter was transferred into 60 ml of secondary antibody solution [solution obtained by dissolving anti-rabbit antibody labeled with horse raddish peroxidase (manufactured by Biorad Co.) with a solution of 1% (W/V) gelatin in TBS buffer to 3000-fold (V/V)]. After shaking at a temperature of 25 °C for 60 minutes, the nitrocellulose filter was washed with 100 ml of Tween-20 washing solution. The procedure described above was repeated twice. The thus obtained nitrocellulose filter was transferred into 120 ml of color forming solution [solution obtained by mixing a solution of 60 mg of 4-chloro-1-naphthol in 20 ml of cold methanol and a solution of 60 µl of 30% (V/V) hydrogen peroxide aqueous solution in 100 ml of TBS buffer] to form a color at a temperature of 25 °C for 10 minutes.

As such, similar procedures were performed on 4 groups, with 96 colonies per one group. In the two groups, protein band stained with luciferase anti-serum was recognized. Next, 96 colonies belonging to the two groups were divided into 8 groups with 12 colonies each and similar procedure was repeated. A protein

that reacted with anti-luciferase serum was noted in one group. Finally, with respect to the 12 colonies contained in this group, each colony was treated in a similar manner, whereby a protein-producing colony that reacted with luciferase anti-serum was identified. By the foregoing procedure, 2 colonies containing luciferase c-DNA were obtained. From the two colonies, plasmid DNA was produced by the method described in Item 5. The obtained recombinant plasmid DNAs were named pALf2B8 and PALF3A6, respectively.

9. Survey of large luciferase c-DNA - Production of c-DNA probe

In 330 µl of TE buffer was dissolved 100 µg of recombinant plasmid, pALf3A6 DNA. To the solution were added 40 µl of Low buffer [100 mM Tris-hydrochloride buffer (pH 7.5)/100 mM $MgCl_2$/10 mM dithiothreitol], 130 units of Pst I (manufactured by Takara Shuzo Co., Ltd.) and 120 units of Sac I (manufactured by Boehringer Mannheim GmbH) to effect cleavage at a temperature of 37 °C for 1.5 hours.

The whole amount of DNA was separated by electrophoresis using 0.7% (W/V) agarose gel. The agarose gel electrophoresis was carried out in accordance with the method of T. Maniatis et al., Molecular Cloning, pages 156-161, Cold Spring Harbor Laboratory (1984)]. DNA band containing luciferase c-DNA was excised and put in a dialysis tube. After 2 ml of TE buffer was supplemented, the dialysis tube was sealed and DNA was eluted from the gel into the buffer by electrophoresis. An equivalent volume of water-saturated phenol was added to this solution. After agitation, the aqueous phase was recovered and DNA was recovered by precipitation with ethanol in a conventional manner.

10 µg of the obtained DNA fragment was dissolved in TE buffer and 16 µl of Med buffer and 64 units of Sau 3 AI (manufactured by Takara Shuzo Co., Ltd.) were added to the solution. After reacting at a temperature of 37 °C for 2 hours, the whole amount was subjected to electrophoresis using 5% (W/V) polyacrylamide gel thereby to isolate DNA fragments. The polyacrylamide gel electrophoresis was carrried out in accordance with the method of A. Maxam [Methods in Enzymology, 65, 506 (1980)]. DNA fragment of 190 bp was isolated by the method as described above to give 1 µg of Sau3 AI luciferase c-DNA fragment.

Using [α-^{32}P] dCTP (manufactured by Amersham Co.), 1 µg of this luciferase c-DNA was labeled according to the nick translation method. The nick translation method was performed using a kit manufactured by Takara Shuzo Co., Ltd. in accordance with the method described in J. Mol. Biol., 113, 237-251 (1977) and Molecular Cloning, pages 109-112, Cold Spring Harbor Laboratory (1982).

10. Survey of large luciferase c-DNA - Colony hybridization

Using as a probe the luciferase c-DNA fargment labelled with ^{32}P produced by the method described above, c-DNA bank of the posterior portion of Photinus pyralis wherein recombinant plasmid pUC19 DNA was a vector was surveyed by colony hybridization [(Protein, Nucleic Acid and Enzyme, 26, 575-579 (1981)] to give colonies having luciferase c-DNA. Recombinant plasmid DNA possessed by one of the colonies was named pALf3 and plasmid DNA was produced by the method described in Item 5. E. coli containing the recombinant plasmid DNA was named E. coli DH 1 (pALf3). The transformant has been deposited as ATCC 67462.

The recombinant plasmid pALf3 DNA described above was subjected to single digestion and double digestion using Xba I, Hind III, BamH I, Eco RI and Pst I (all manufactured by Takara Shuzo Co., Ltd.). The obtained DNA fragments were analyzed by agarose gel electrophoresis on mobility pattern. By comparing the obtained mobility pattern with standard mobility pattern of DNA fragment obtained by digesting λ phage DNA (manufactured by Takara Shuzo Co., Ltd.) with Hind III, the size of the c-DNA inserted in pALf3 was turned out to be 1,700 bp. A restriction enzyme map of the plasmid described above is shown in Fig. 3.

11. Preparation of m-RNA of Luciola cruciata

Ten grams of living Luciola cruciata (GENJI firefly, purchased from Seibu Department Store) were put in a ultra-low temperature freezer box and frozen. Each posterior portion was cut off with scissors. To 2 g of the obtained posterior portion was added 18 ml of guanidine isothiocyanate solution. According to the method described in Item 1, 1.1 mg of RNA was prepared. In accordance with the method described in Item 1, 1.1 mg of this RNA was subjected to column chromatography of oligo (dT)-cellulose to obtain 30 µg of m-RNA for the posterior portion of Luciola cruciata.

12. Production of c-DNA bank of Luciola cruciata posterior portion

Synthesis of c-DNA was performed using a kit purchased from Amersham Co. in accordance with the method indicated by Amersham Co. which is described in Mol. Cell Biol., 2, 161 (1982) and Gene, 25, 263 (1983).

From 2 µg of the Luciola cruciata posterior portion RNA, 0.9 µg of double stranded c-DNA was synthesized. Using the method described in Item 4, a tail of polydeoxycytidine was added to 0.3 µg of this c-DNA.

This c-DNA, 20 ng, and 500 ng of pUC19 plasmid produced in Item 6, wherein a polyguanosine tail had been added to the Pst I site thereof, were annealed in accordance with the method described in Item 7. E. coli DH 1 strain (ATCC 33849) was transformed by annealed DNA by the method of Hanahan [DNA Cloning, 1, 109-135 (1985)] thereby to produce c-DNA bank of Luciola cruciata tail.

13. Survey of luciferase c-DNA derived from Luciola cruciata

In 90 µl of TE buffer was dissolved 10 µg of recombinant plasmid pALf3 DNA obtained in Item 10 and, 10 µl of Med buffer, 25 units of restriction enzyme Eco RI and 25 units of restriction enzyme Cla I (both manufactured by Takara Shuzo Co., Ltd.) were added to the solution. The reaction was performed at a temperature of 37 °C for 2 hours to cleave DNA. From the cleaved recombinant plasmid pALf3 DNA, 800 bp of Eco RI/Cla I DNA fragment containing luciferase c-DNA derived from Photinus pyralis (American firefly) was isolated in accordance with the method described in Item 9 using agarose gel electrophoresis. Thus, 1 µg of Eco RI/Cla I DNA fragment was obtained. Using [α-^{32}P] dCTP (manufactured by Amersham Co.), 1 µg of this DNA was labelled with ^{32}P in accordance with the nick translation method described in Item 9. Using as a probe the Eco RI/Cla I DNA fragment labeled with ^{32}P, c-DNA bank of the Luciola cruciata posterior portion was surveyed by the colony hybridization described in Item 10 thereby to select E. coli having luciferase c-DNA derived from Luciola cruciata. Several colonies of E. coli capable of hybridizing with the probe were obtained. Recombinant plasmid DNA possessed by one of these colonies was named pGLf1. The recombinant plasmid DNA was isolated in accordance with the method described in Item 5.

The recombinant plasmid pGLf1 DNA described above was subjected to single digestion and double digestion using Hpa I, Hind III, Eco RV, Dra I, Af1 II, Hinc II, Pst I (all manufactured by Takara Shuzo Co., Ltd.) and Ssp I (manufactured by New England Biolab Co.). The obtained DNA fragments were analyzed by agarose gel electrophoresis on mobility pattern. By comparing the obtained mobility pattern with standard mobility pattern of DNA fragment obtained by digesting λ phage DNA (manufactured by Takara Shuzo Co., Ltd.) with Hind III, the size of the c-DNA inserted in pGLf1 was turned out to be 2,000 bp. A restriction enzyme map of the plasmid described above is shown in Fig. 4.

14. Preparation of m-RNA of Luciola lateralis

Five grams of living Luciola lateralis (HEIKE firefly, purchased from Kawahara Choju Trading Co., Ltd.) were put in a ultra-low temperature freezer box and frozen. Each posterior portion was cut off with scissors. To 1 g of the obtained posterior portion was added 18 ml of guanidine isothiocyanate solution. Following the method described in Item 1, 340 µg of RNA was prepared. In accordance with the method described in Item 1, 340 µg of the RNA was subjected to column chromatography of oligo(dT)-cellulose to obtain 6 µg of m-RNA from the posterior portion of Luciola lateralis.

15. Production of c-DNA bank of Luciola lateralis posterior portion

Synthesis of c-DNA was performed using a kit purchased from Amersham Co.

Using 2.0 µg of m-RNA obtained as above, c-DNA was synthesized in accordance with the method indicated by Amersham Co. which is described in Mol. Cell Biol., 2, 161 (1982) and Gene, 25, 263 (1983). As the result, 250 ng of double stranded c-DNA was obtained.

The thus obtained c-DNA, 250 ng, was subjected to methylation at the restriction enzyme Eco RI site using c-DNA cloning kit manufactured by Amersham Co. as instructed by Amersham Co. Furthermore, Eco RI linker was adhered to the both termini of the c-DNA.

After 1 µl of Med buffer [100 mM Tris-hydrochloride buffer (pH 7.5)/100 mM $MgCl_2$/10 mM dithiothreitol/500 mM NaCl] was added to a solution of 100 ng of plasmid pUC119 DNA (manufactured by Takara Shuzo Co., Ltd.) in 8 µl of water, 10 units of restriction enzyme Eco RI (manufactured by Takara Shuzo Co., Ltd.) was further added to the mixture. A cleavage treatment was conducted at a temperature of

37 ° C for an hour.

Subsequently, 1 μl of 1M Tris-hydrochloride buffer (pH 8.0) and 0.3 unit (1 μl) of alkaline phosphatase (manufactured by Takara Shuzo Co., Ltd.) were added to the cleavage product and the mixture was subjected to enzymatic reaction at a temperature of 65 ° C for an hour to effect dephosphorylation of the termini of the cleavage product. After 12 μl of water-saturated phenol was added to the dephosphorylated product to remove protein, 1 μl of 3M sodium acetate (pH 5.8) and 26 μl of cold ethanol were added to the recovered aqueous phase, respectively. The mixture was allowed to stand at a temperature of -70 ° C for 15 minutes. By centrifuging treatment at 12,000 r.p.m. for 5 minutes with a trace centrifuging machine (manufactured by TOMI SEIKO K.K., MRX-150) to recover DNA.

The thus obtained DNA was cleaved with restriction enzyme Eco RI and its termini were dephospholyated. The resulting plasmid vector pUC119 DNA, 100 ng, was mixed with 250 ng of c-DNA produced in Item 15. After the mixutre was suspended in 8 μl of water, 1 μl of ligation buffer [200 mM $MgCl_2$/660 mM Tris-hydrochloride buffer (pH 7.6)/10 mM ATP/150 mM dithiothreitol] was added to the resulting mixture. Furthermore, 1 unit of T4 DNA ligase (manufactured by Takara Shuzo Co., Ltd.) was added thereto and the mixture was allowed to stand at a temperature of 16 ° C for 16 hours, whereby ligation of the plasmid vector and c-DNA was performed to give the reaction product.

Using this reaction product, E. coli DH1 (ATCC 33849) strain was transformed by the method of Hanahan [DNA Cloning, 1, 109-135 (1985)] to produce a c-DNA bank derived from the posterior portion of Luciola lateralis containing plasmid pUC119 DNA as a vector.

16. Survey of luciferase c-DNA derived from Luciola lateralis

In 90 μl of TE buffer was dissolved 10 μg of recombinant plasmid pGLf1 DNA obtained in Item 13 and, 10 μl of Med buffer, 25 units of restriction enzyme Pst I (manufactured by Takara Shuzo Co., Ltd.) was added to the solution. The reaction was performed at a temperature of 37 ° C for 2 hours to cleave DNA. From the cleaved recombinant plasmid pGLf1 DNA, 2,000 bp of Pst I DNA fragment containing Luciola cruciata-derived luciferase c-DNA portion was isolated in accordance with the method described in Item 9 using agarose gel electrophoresis. Thus, 1 μg of Pst I DNA fragment was obtained. Using [α-^{32}P] dCTP (manufactured by Amersham Co.), 1 μg of this DNA was labelled with ^{32}P in accordance with the nick translation method described in Item 9. Using as a probe the Pst I DNA fragment labeled with ^{32}P, c-DNA bank of the Luciola lateralis posterior portion was surveyed by the colony hybridization described in Item 10 thereby to select E. coli bearing luciferase c-DNA derived from Luciola lateralis. Several colonies of E. coli capable of hybridizing with the probe were obtained. Plasmid DNA possessed by one of these colonies was named pHLf7. The recombinant plasmid DNA was isolated in accordance with the method described in Item 5.

The thus obtained E. coli DH1 (pHLf7) has been deposited in the Fermentation Research Institute, the Agency of Industrial Science and Technology, Japan, under the Budapest Treaty with the accession number FERM BP-1917.

The recombinant plasmid pHLf7 DNA described above was subjected to single digestion and double digestion using Hpa I, Eco RV, Apa I, Hind III and Eco RI (all manufactured by Takara Shuzo Co., Ltd.) and Ssp I (manufactured by New England Biolab Co.). The obtained DNA fragments were analyzed by agarose gel electrophoresis on mobility pattern. By comparing the obtained mobility pattern with standard mobility pattern of DNA fragment obtained by digesting λ phage DNA (manufactured by Takara Shuzo Co., Ltd.) with Hind III, the size of the gene encoding Luciola lateralis-derived luciferase was turned out to be 2,000 bp. A restriction enzyme map of the plasmid described above is shown in Fig. 5.

To a solution of 10 μg of recombinant plasmid pHLf7 DNA in 45 μl of TE buffer were added 5 μl of Med buffer and 2 units of restriction enzyme Eco RI (manufactured by Takara Shuzo Co., Ltd.), respectively. The mixture was reacted at a temperature of 37 ° C for 2 hours to give a partial digestion product of DNA.

Then, the partial digestion product was subjected to agarose gel electrophoresis described in Item 9 and 1 μg of Eco RI fragment of 2,000 bp containing all the gene encoding Luciola lateralis-derived luciferase was isolated.

17. Cultivation of E. coli DH1 (pHLf7) (FERM BP-1917) and production of crude enzyme solution

E. coli DH1 (pHLf7) (FERM BP-1917) was shake cultured in 3 ml of LB-amp medium [1% (W/V) bactotrypton, 0.5% (W/V) yeast extract, 0.5% (W/V) NaCl and ampicillin (50 μg/ml)] at a temperature of 37 ° C for 18 hours. This culture solution, 0.5 ml, was inoculated on 10 μl of the aforesaid LB-amp medium

and 1 mM isopropyl-β-D-thiogalactoside was added thereto. After shake culture at a temperature of 37°C for 4 hours, the culture was subjected to a centrifuging operation at 8,000 r.p.m. for 10 minutes to give 20 mg of wet cells.

The recovered cells were suspended in 0.9 ml of a buffer composed of 0.1 M KH_2PO_4 (pH 7.8), 2 mM EDTA, 1 mM dithiothreitol and 0.2 mg/ml protamine sulfate. Further 100 µl of 10 mg/ml lysozyme solution was supplemented to the suspension. The mixture was allowed to stand in ice for 15 minutes. Next, the suspension was frozen in methanol-dry ice bath and then allowed to stand at a temperature of 25°C to completely thaw. Further by performing a centrifuging operation at 12,000 r.p.m. for 5 minutes, 1 ml of crude enzyme solution was obtained as the supernatant.

The luciferase activity in the thus obtained crude enzyme solution was performed by the method described below. The results are shown in Table 1 below.

The measurement of luciferase activity in the crude enzyme solution obtained was performed by counting the number of photons generated in accordance with the method of Kricka [Archives of Biochemistry and Biophysics, 217, 674 (1982)].

That is, 260 µl of 25 mM glycylglycine buffer (pH 7.8), 16 µl of 0.1 M magnesium sulfate and 24 µl of 1 mM luciferine (manufactured by Sigma Inc.) and 10 µl of the crude enzyme solution were mixed. Then 100 µl of 20 mM ATP was added to the mixture. The number of photons generated was integrated for 20 seconds. The integrated values are shown in Table 1 below. For purpose of comparison, a luciferase activity was measured also with plasmid pUC119 DNA-bearing E. coli DH1 strain [E. coli DH1 (pUC119)]. The results are also shown in Table 1 below.

Table 1

Sample \ Item	Number of Photon/ml Culture Solution
E. coli DH1 (pHLf7) (invention)	6.2×10^6
E. coli DH1 (pUC119) (control)	1.0×10^4

As is clear from the table above, it is noted that the count of photons increased in E. coli DH1 (pHLf7) bearing the recombinant plasmid pHLf7 containing the luciferase gene of this invention as compared to the control and therefore, luciferase is produced in the cells of E. coli used in this invention.

18. Analysis of nucleotide sequence of luciferase c-DNA derived from Luciola lateralis

Recombinant plasmid pHLf7 DNA, 10 µg, was cleaved with restriction enzyme Eco RI (manufactured by Takara Shuzo Co., Ltd.) to give 2.0 µg of 1.7 Kb DNA fragment and 0.5 µg of 0.3 Kb DNA fragment, containing luciferase c-DNA. These DNA fragments were subcloned at the Eco RI site of plasmid pUC118 DNA (manufactured by Takara Shuzo Co., Ltd.) to give 4 plasmids, pHLf11, pHLf12, pHLf13 and pHLf14, based on differences in kind of the inserted fragments (1.7 Kb and 0.3 Kb) and in orientation of the insertion (the 1.7 Kb fragment was subcloned to pHLf11 and pHLf12, and the 0.3 Kb fragment was subcloned to pHLf13 and pHLf14).

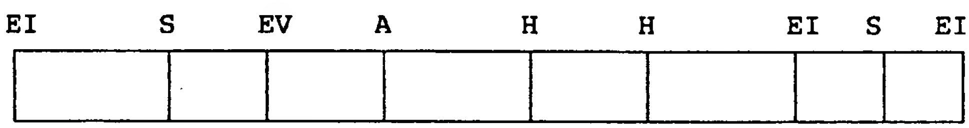
Cleavage treatment of the recombinant plasmids pHLf7 DNA and plasmid pUC118 DNA with Eco RI (method described in Item 6), isolation of the luciferase c-DNA fragment using agarose gel electrophoresis (method described in Item 9), ligation of plasmid pUC119 DNA and the luciferase c-DNA fragment (method described in Item 5), transformation of E. coli JM101 strain (ATCC 33876) using ligation reaction liquid (method described in Item 5) and production of recombinant plasmids pHLf11, pHLf12, pHLf13 and pHLf14 (method described in Item 5) followed the methods described within parentheses.

Next, using the recombinant plasmid DNAs pHLf11, pHLf12, pHLf13 and pHLf14, plasmid DNAs wherein various deletions were introduced into the luciferase c-DNA were produced using a deletion kit for

killosequence (manufactured by Takara Shuzo Co., Ltd.) in accordance with the method of Henikoff [Gene, 28, 351-359 (1984)]. These plasmid DNAs were introduced into E. coli JM101 strain (ATCC 33876) by the method described in Item 5. By infecting the thus obtained E. coli with helper phage M13K07 (manufactured by Takara Shuzo Co., Ltd.), single strand DNA was produced in accordance with the method of Messing [Methods in Enzymology, 101, 20-78 (1983)]. Sequencing with the obtained single strand DNA was carried out by the method of Messing described above, using M13 sequencing kit (manufactured by Takara Shuzo Co., Ltd.). Gel electrophoresis for analyzing a nucleotide sequence was carried out using 8% (W/V) polyacrylamide gel (manufactured by Fuji Photo Film Co., Ltd.). The nucleotide sequence of the Luciola lateralis-derived luciferase-coding c-DNA obtained is shown in Fig. 6. Fig. 7 shows an amino acid sequence of polypeptide translated from the c-DNA and Fig. 8 shows a sequence corresponding to c-DNA in the amino acid sequence.

Claims

1. A Luciola lateralis-derived luciferase gene defined by a restriction enzyme map described below:



wherein EI represents Eco RI, S represents Ssp I, EV represents Eco RV, A represents Apa I and H represents Hpa I.

2. A luciferase gene according to claim 1, which encodes an amino acid sequence shown below:

```
                                             10
Met Glu Asn Met Glu Asn Asp Glu Asn Ile
                                             20
Val Tyr Gly Pro Glu Pro Phe Tyr Pro Ile
                                             30
Glu Glu Gly Ser Ala Gly Ala Gln Leu Arg
                                             40
Lys Tyr Met Asp Arg Tyr Ala Lys Leu Gly
                                             50
Ala Ile Ala Phe Thr Asn Ala Leu Thr Gly
                                             60
Val Asp Tyr Thr Tyr Ala Glu Tyr Leu Glu
                                             70
Lys Ser Cys Cys Leu Gly Glu Ala Leu Lys
                                             80
Asn Tyr Gly Leu Val Val Asp Gly Arg Ile
                                             90
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe
                                            100
Phe Ile Pro Val Leu Ala Gly Leu Phe Ile
                                            110
Gly Val Gly Val Ala Pro Thr Asn Glu Ile
                                            120
Tyr Thr Leu Arg Glu Leu Val His Ser Leu
                                            130
Gly Ile Ser Lys Pro Thr Ile Val Phe Ser
                                            140
Ser Lys Lys Gly Leu Asp Lys Val Ile Thr
                                            150
Val Gln Lys Thr Val Thr Ala Ile Lys Thr
                                            160
Ile Val Ile Leu Asp Ser Lys Val Asp Tyr
                                            170
Arg Gly Tyr Gln Ser Met Asp Asn Phe Ile
                                            180
Lys Lys Asn Thr Pro Gln Gly Phe Lys Gly
                                            190
Ser Ser Phe Lys Thr Val Glu Val Asn Arg
```

									200
Lys	Glu	Gln	Val	Ala	Leu	Ile	Met	Asn	Ser
									210
Ser	Gly	Ser	Thr	Gly	Leu	Pro	Lys	Gly	Val
									220
Gln	Leu	Thr	His	Glu	Asn	Ala	Val	Thr	Arg
									230
Phe	Ser	His	Ala	Arg	Asp	Pro	Ile	Tyr	Gly
									240
Asn	Gln	Val	Ser	Pro	Gly	Thr	Ala	Ile	Leu
									250
Thr	Val	Val	Pro	Phe	His	His	Gly	Phe	Gly
									260
Met	Phe	Thr	Thr	Leu	Gly	Tyr	Leu	Thr	Cys
									270
Gly	Phe	Arg	Ile	Val	Met	Leu	Thr	Lys	Phe
									280
Asp	Glu	Glu	Thr	Phe	Leu	Lys	Thr	Leu	Gln
									290
Asp	Tyr	Lys	Cys	Ser	Ser	Val	Ile	Leu	Val
									300
Pro	Thr	Leu	Phe	Ala	Ile	Leu	Asn	Arg	Ser
									310
Glu	Leu	Leu	Asp	Lys	Tyr	Asp	Leu	Ser	Asn
									320
Leu	Val	Glu	Ile	Ala	Ser	Gly	Gly	Ala	Pro
									330
Leu	Ser	Lys	Glu	Ile	Gly	Glu	Ala	Val	Ala
									340
Arg	Arg	Phe	Asn	Leu	Pro	Gly	Val	Arg	Gln
									350
Gly	Tyr	Gly	Leu	Thr	Glu	Thr	Thr	Ser	Ala
									360
Ile	Ile	Ile	Thr	Pro	Glu	Gly	Asp	Asp	Lys
									370
Pro	Gly	Ala	Ser	Gly	Lys	Val	Val	Pro	Leu
									380
Phe	Lys	Ala	Lys	Val	Ile	Asp	Leu	Asp	Thr

									390
Lys	Lys	Thr	Leu	Gly	Pro	Asn	Arg	Arg	Gly
									400
Glu	Val	Cys	Val	Lys	Gly	Pro	Met	Leu	Met
									410
Lys	Gly	Tyr	Val	Asp	Asn	Pro	Glu	Ala	Thr
									420
Arg	Glu	Ile	Ile	Asp	Glu	Glu	Gly	Trp	Leu
									430
His	Thr	Gly	Asp	Ile	Gly	Tyr	Tyr	Asp	Glu
									440
Glu	Lys	His	Phe	Phe	Ile	Val	Asp	Arg	Leu
									450
Lys	Ser	Leu	Ile	Lys	Tyr	Lys	Gly	Tyr	Gln
									460
Val	Pro	Pro	Ala	Glu	Leu	Glu	Ser	Val	Leu
									470
Leu	Gln	His	Pro	Asn	Ile	Phe	Asp	Ala	Gly
									480
Val	Ala	Gly	Val	Pro	Asp	Pro	Ile	Ala	Gly
									490
Glu	Leu	Pro	Gly	Ala	Val	Val	Val	Leu	Glu
									500
Lys	Gly	Lys	Ser	Met	Thr	Glu	Lys	Glu	Val
									510
Met	Asp	Tyr	Val	Ala	Ser	Gln	Val	Ser	Asn
									520
Ala	Lys	Arg	Leu	Arg	Gly	Gly	Val	Arg	Phe
									530
Val	Asp	Glu	Val	Pro	Lys	Gly	Leu	Thr	Gly
									540
Lys	Ile	Asp	Gly	Lys	Ala	Ile	Arg	Glu	Ile
Leu	Lys	Lys	Pro	Val	Ala	Lys	Met		

3. A luciferase gene according to Claim 1 or 2 which is represented by a nucleotide sequence shown below.

30
ATG GAA AAC ATG GAG AAC GAT GAA AAT ATT

60
GTG TAT GGT CCT GAA CCA TTT TAC CCT ATT

90
GAA GAG GGA TCT GCT GGA GCA CAA TTG CGC

120
AAG TAT ATG GAT CGA TAT GCA AAA CTT GGA

150
GCA ATT GCT TTT ACT AAC GCA CTT ACC GGT

180
GTC GAT TAT ACG TAC GCC GAA TAC TTA GAA

210
AAA TCA TGC TGT CTA GGA GAG GCT TTA AAG

240
AAT TAT GGT TTG GTT GTT GAT GGA AGA ATT

270
GCG TTA TGC AGT GAA AAC TGT GAA GAA TTC

300
TTT ATT CCT GTA TTA GCC GGT TTA TTT ATA

330
GGT GTC GGT GTG GCT CCA ACT AAT GAG ATT

360
TAC ACT CTA CGT GAA TTG GTT CAC AGT TTA

390
GGC ATC TCT AAG CCA ACA ATT GTA TTT AGT

420
TCT AAA AAA GGA TTA GAT AAA GTT ATA ACT

450
GTA CAA AAA ACG GTA ACT GCT ATT AAA ACC

480
ATT GTT ATA TTG GAC AGC AAA GTG GAT TAT

510
AGA GGT TAT CAA TCC ATG GAC AAC TTT ATT

540
AAA AAA AAC ACT CCA CAA GGT TTC AAA GGA

570
TCA AGT TTT AAA ACT GTA GAA GTT AAC CGC

AAA GAA CAA GTT GCT CTT ATA ATG AAC TCT 600
TCG GGT TCA ACC GGT TTG CCA AAA GGT GTG 630
CAA CTT ACT CAT GAA AAT GCA GTC ACT AGA 660
TTT TCT CAC GCT AGA GAT CCA ATT TAT GGA 690
AAC CAA GTT TCA CCA GGC ACG GCT ATT TTA 720
ACT GTA GTA CCA TTC CAT CAT GGT TTT GGT 750
ATG TTT ACT ACT TTA GGC TAT CTA ACT TGT 780
GGT TTT CGT ATT GTC ATG TTA ACG AAA TTT 810
GAC GAA GAG ACT TTT TTA AAA ACA CTG CAA 840
GAT TAC AAA TGT TCA AGC GTT ATT CTT GTA 870
CCG ACT TTG TTT GCA ATT CTT AAT AGA AGT 900
GAA TTA CTC GAT AAA TAT GAT TTA TCA AAT 930
TTA GTT GAA ATT GCA TCT GGC GGA GCA CCT 960
TTA TCT AAA GAA ATT GGT GAA GCT GTT GCT 990
AGA CGT TTT AAT TTA CCG GGT GTT CGT CAA 1020
GGC TAT GGT TTA ACA GAA ACA ACC TCT GCA 1050
ATT ATT ATC ACA CCG GAA GGC GAT GAT AAA 1080
CCA GGT GCT TCT GGC AAA GTT GTG CCA TTA 1110
TTT AAA GCA AAA GTT ATC GAT CTT GAT ACT 1140

```
                                              1170
AAA AAA ACT TTG GGC CCG AAC AGA CGT GGA
                                              1200
GAA GTT TGT GTA AAG GGT CCT ATG CTT ATG
                                              1230
AAA GGT TAT GTA GAT AAT CCA GAA GCA ACA
                                              1260
AGA GAA ATC ATA GAT GAA GAA GGT TGG TTG
                                              1290
CAC ACA GGA GAT ATT GGG TAT TAC GAT GAA
                                              1320
GAA AAA CAT TTC TTT ATC GTG GAT CGT TTG
                                              1350
AAG TCT TTA ATC AAA TAC AAA GGA TAT CAA
                                              1380
GTA CCA CCT GCT GAA TTA GAA TCT GTT CTT
                                              1410
TTG CAA CAT CCA AAT ATT TTT GAT GCC GGC
                                              1440
GTT GCT GGC GTT CCA GAT CCT ATA GCT GGT
                                              1470
GAG CTT CCG GGA GCT GTT GTT GTA CTT GAA
                                              1500
AAA GGA AAA TCT ATG ACT GAA AAA GAA GTA
                                              1530
ATG GAT TAC GTT GCT AGT CAA GTT TCA AAT
                                              1560
GCA AAA CGT TTG CGT GGT GGT GTC CGT TTT
                                              1590
GTG GAC GAA GTA CCT AAA GGT CTC ACT GGT
                                              1620
AAA ATT GAC GGT AAA GCA ATT AGA GAA ATA
CTG AAG AAA CCA GTT GCT AAG ATG
```

4. A novel recombinant DNA comprising a vector DNA into which a gene coding for Luciola lateralis-derived luciferase according to claims 1-3 is inserted.

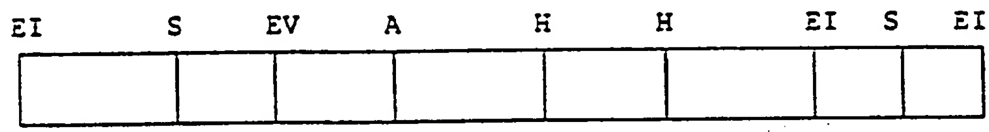
5. A novel recombinant DNA according to claim 4, wherein said vector DNA is plasmid pUC119.

6. A method for producing a luciferase which comprises culturing in a medium a microorganism belonging to the genus Escherichia and bearing a recombinant DNA according to claim 4 obtained by inserting a gene coding for Luciola lateralis-derived luciferase into a vector DNA and collecting said luciferase from the culture.

7. A method for producing a luciferase according to claim 6, wherein said vector DNA is plasmid pUC119.

Patentansprüche

1. Ein von Luciola lateralis abgeleitetes Luciferase-Gen, definiert durch die nachfolgend angegebene Restriktionsenzymkarte:



worin EI Eco RI bedeutet, S Ssp I bedeutet, EV Eco RV bedeutet, A Apa I bedeutet und H Hpa I bedeutet.

2. Luciferase-Gen nach Anspruch 1, dadurch gekennzeichnet, daß es die nachfolgend angegebene Aminosäuresequenz codiert:

```
                                                10
Met Glu Asn Met Glu Asn Asp Glu Asn Ile
                                                20
Val Tyr Gly Pro Glu Pro Phe Tyr Pro Ile
                                                30
Glu Glu Gly Ser Ala Gly Ala Gln Leu Arg
                                                40
Lys Tyr Met Asp Arg Tyr Ala Lys Leu Gly
                                                50
Ala Ile Ala Phe Thr Asn Ala Leu Thr Gly
                                                60
Val Asp Tyr Thr Tyr Ala Glu Tyr Leu Glu
                                                70
Lys Ser Cys Cys Leu Gly Glu Ala Leu Lys
```

80
Asn Tyr Gly Leu Val Val Asp Gly Arg Ile
90
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe
100
Phe Ile Pro Val Leu Ala Gly Leu Phe Ile
110
Gly Val Gly Val Ala Pro Thr Asn Glu Ile
120
Tyr Thr Leu Arg Glu Leu Val His Ser Leu
130
Gly Ile Ser Lys Pro Thr Ile Val Phe Ser
140
Ser Lys Lys Gly Leu Asp Lys Val Ile Thr
150
Val Gln Lys Thr Val Thr Ala Ile Lys Thr
160
Ile Val Ile Leu Asp Ser Lys Val Asp Tyr
170
Arg Gly Tyr Gln Ser Met Asp Asn Phe Ile
180
Lys Lys Asn Thr Pro Gln Gly Phe Lys Gly
190
Ser Ser Phe Lys Thr Val Glu Val Asn Arg
200
Lys Glu Gln Val Ala Leu Ile Met Asn Ser
210
Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
220
Gln Leu Thr His Glu Asn Ala Val Thr Arg
230
Phe Ser His Ala Arg Asp Pro Ile Tyr Gly
240
Asn Gln Val Ser Pro Gly Thr Ala Ile Leu
250
Thr Val Val Pro Phe His His Gly Phe Gly
260
Met Phe Thr Thr Leu Gly Tyr Leu Thr Cys
270
Gly Phe Arg Ile Val Met Leu Thr Lys Phe
280
Asp Glu Glu Thr Phe Leu Lys Thr Leu Gln

290
Asp Tyr Lys Cys Ser Ser Val Ile Leu Val
300
Pro Thr Leu Phe Ala Ile Leu Asn Arg Ser
310
Glu Leu Leu Asp Lys Tyr Asp Leu Ser Asn
320
Leu Val Glu Ile Ala Ser Gly Gly Ala Pro
330
Leu Ser Lys Glu Ile Gly Glu Ala Val Ala
340
Arg Arg Phe Asn Leu Pro Gly Val Arg Gln
350
Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala
360
Ile Ile Ile Thr Pro Glu Gly Asp Asp Lys
370
Pro Gly Ala Ser Gly Lys Val Val Pro Leu
380
Phe Lys Ala Lys Val Ile Asp Leu Asp Thr
390
Lys Lys Thr Leu Gly Pro Asn Arg Arg Gly
400
Glu Val Cys Val Lys Gly Pro Met Leu Met
410
Lys Gly Tyr Val Asp Asn Pro Glu Ala Thr
420
Arg Glu Ile Ile Asp Glu Glu Gly Trp Leu
430
His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu
440
Glu Lys His Phe Phe Ile Val Asp Arg Leu
450
Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
460
Val Pro Pro Ala Glu Leu Glu Ser Val Leu
470
Leu Gln His Pro Asn Ile Phe Asp Ala Gly
480
Val Ala Gly Val Pro Asp Pro Ile Ala Gly
490
Glu Leu Pro Gly Ala Val Val Val Leu Glu
500
Lys Gly Lys Ser Met Thr Glu Lys Glu Val

```
                                                510
Met Asp Tyr Val Ala Ser Gln Val Ser Asn
                                                520
Ala Lys Arg Leu Arg Gly Gly Val Arg Phe
                                                530
Val Asp Glu Val Pro Lys Gly Leu Thr Gly
                                                540
Lys Ile Asp Gly Lys Ala Ile Arg Glu Ile

Leu Lys Lys Pro Val Ala Lys Met
```

3. Luciferase-Gen gemäß Anspruch 1 oder 2, dadurch gekennzeichnet, daß es durch die nachfolgend angegebene Nucleotidsequenz dargestellt wird.

```
                                                 30
ATG GAA AAC ATG GAG AAC GAT GAA AAT ATT
                                                 60
GTG TAT GGT CCT GAA CCA TTT TAC CCT ATT
                                                 90
GAA GAG GGA TCT GCT GGA GCA CAA TTG CGC
                                                120
AAG TAT ATG GAT CGA TAT GCA AAA CTT GGA
                                                150
GCA ATT GCT TTT ACT AAC GCA CTT ACC GGT
                                                180
GTC GAT TAT ACG TAC GCC GAA TAC TTA GAA
                                                210
AAA TCA TGC TGT CTA GGA GAG GCT TTA AAG
                                                240
AAT TAT GGT TTG GTT GTT GAT GGA AGA ATT
                                                270
GCG TTA TGC AGT GAA AAC TGT GAA GAA TTC
                                                300
TTT ATT CCT GTA TTA GCC GGT TTA TTT ATA
                                                330
GGT GTC GGT GTG GCT CCA ACT AAT GAG ATT
```

```
                                                 360
TAC ACT CTA CGT GAA TTG GTT CAC AGT TTA
                                                 390
GGC ATC TCT AAG CCA ACA ATT GTA TTT AGT
                                                 420
TCT AAA AAA GGA TTA GAT AAA GTT ATA ACT
                                                 450
GTA CAA AAA ACG GTA ACT GCT ATT AAA ACC
                                                 480
ATT GTT ATA TTG GAC AGC AAA GTG GAT TAT
                                                 510
AGA GGT TAT CAA TCC ATG GAC AAC TTT ATT
                                                 540
AAA AAA AAC ACT CCA CAA GGT TTC AAA GGA
                                                 570
TCA AGT TTT AAA ACT GTA GAA GTT AAC CGC
                                                 600
AAA GAA CAA GTT GCT CTT ATA ATG AAC TCT
                                                 630
TCG GGT TCA ACC GGT TTG CCA AAA GGT GTG
                                                 660
CAA CTT ACT CAT GAA AAT GCA GTC ACT AGA
                                                 690
TTT TCT CAC GCT AGA GAT CCA ATT TAT GGA
                                                 720
AAC CAA GTT TCA CCA GGC ACG GCT ATT TTA
                                                 750
ACT GTA GTA CCA TTC CAT CAT GGT TTT GGT
                                                 780
ATG TTT ACT ACT TTA GGC TAT CTA ACT TGT
                                                 810
GGT TTT CGT ATT GTC ATG TTA ACG AAA TTT
                                                 840
GAC GAA GAG ACT TTT TTA AAA ACA CTG CAA
                                                 870
GAT TAC AAA TGT TCA AGC GTT ATT CTT GTA
                                                 900
CCG ACT TTG TTT GCA ATT CTT AAT AGA AGT
                                                 930
GAA TTA CTC GAT AAA TAT GAT TTA TCA AAT
                                                 960
TTA GTT GAA ATT GCA TCT GGC GGA GCA CCT
```

```
                                                990
TTA TCT AAA GAA ATT GGT GAA GCT GTT GCT
                                               1020
AGA CGT TTT AAT TTA CCG GGT GTT CGT CAA
                                               1050
GGC TAT GGT TTA ACA GAA ACA ACC TCT GCA
                                               1080
ATT ATT ATC ACA CCG GAA GGC GAT GAT AAA
                                               1110
CCA GGT GCT TCT GGC AAA GTT GTG CCA TTA
                                               1140
TTT AAA GCA AAA GTT ATC GAT CTT GAT ACT
                                               1170
AAA AAA ACT TTG GGC CCG AAC AGA CGT GGA
                                               1200
GAA GTT TGT GTA AAG GGT CCT ATG CTT ATG
                                               1230
AAA GGT TAT GTA GAT AAT CCA GAA GCA ACA
                                               1260
AGA GAA ATC ATA GAT GAA GAA GGT TGG TTG
                                               1290
CAC ACA GGA GAT ATT GGG TAT TAC GAT GAA
                                               1320
GAA AAA CAT TTC TTT ATC GTG GAT CGT TTG
                                               1350
AAG TCT TTA ATC AAA TAC AAA GGA TAT CAA
                                               1380
GTA CCA CCT GCT GAA TTA GAA TCT GTT CTT
                                               1410
TTG CAA CAT CCA AAT ATT TTT GAT GCC GGC
                                               1440
GTT GCT GGC GTT CCA GAT CCT ATA GCT GGT
                                               1470
GAG CTT CCG GGA GCT GTT GTT GTA CTT GAA
                                               1500
AAA GGA AAA TCT ATG ACT GAA AAA GAA GTA
                                               1530
ATG GAT TAC GTT GCT AGT CAA GTT TCA AAT
                                               1560
GCA AAA CGT TTG CGT GGT GGT GTC CGT TTT
```

```
                                        1590
GTG GAC GAA GTA CCT AAA GGT CTC ACT GGT
                                        1620
AAA ATT GAC GGT AAA GCA ATT AGA GAA ATA

CTG AAG AAA CCA GTT GCT AAG ATG
```

4. Neue rekombinante DNA, umfassend eine Vektor-DNA, in die ein Gen gemäß den Ansprüchen 1 bis 3, das für von Luciola lateralis abgeleitete Luciferase codiert, eingeführt ist.

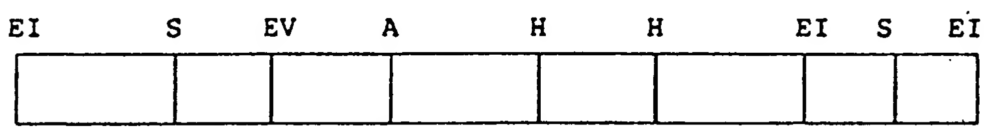
5. Neue rekombinante DNA gemäß Anspruch 4, dadurch gekennzeichent, daß die Vektor-DNA Plasmid pUC119 ist.

6. Verfahren zur Herstellung einer Luciferase, dadurch gekennzeichnet, daß man in einem Medium einen Mikroorganismus der Gattung Escherichia, der eine rekombinante DNA gemäß Anspruch 4 trägt, die erhalten wurde durch Einführung eines Gens, das für von Luciola lateralis abgeleitete Luciferase codiert, in eine Vektor-DNA kultiviert und die Luciferase aus der Kultur gewinnt.

7. Verfahren zur Herstellung einer Luciferase gemäß Anspruch 6, dadurch gekennzeichnet, daß die Vektor-DNA Plasmid pUC119 ist.

Revendications

1. Gène de luciférase dérivé de Luciola lateralis, défini par la carte de restriction décrite ci-dessous:



dans laquelle EI représente EcoRI, S représente Ssp I, EV représente Eco RV, A représente Apa I et H représente Hpa I.

2. Gène de luciférase selon la revendication 1, qui code la séquence d'acides aminés présentée ci-dessous:

10
Met Glu Asn Met Glu Asn Asp Glu Asn Ile
20
Val Tyr Gly Pro Glu Pro Phe Tyr Pro Ile
30
Glu Glu Gly Ser Ala Gly Ala Gln Leu Arg
40
Lys Tyr Met Asp Arg Tyr Ala Lys Leu Gly
50
Ala Ile Ala Phe Thr Asn Ala Leu Thr Gly
60
Val Asp Tyr Thr Tyr Ala Glu Tyr Leu Glu
70
Lys Ser Cys Cys Leu Gly Glu Ala Leu Lys
80
Asn Tyr Gly Leu Val Val Asp Gly Arg Ile
90
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe

100
Phe Ile Pro Val Leu Ala Gly Leu Phe Ile
110
Gly Val Gly Val Ala Pro Thr Asn Glu Ile
120
Tyr Thr Leu Arg Glu Leu Val His Ser Leu
130
Gly Ile Ser Lys Pro Thr Ile Val Phe Ser
140
Ser Lys Lys Gly Leu Asp Lys Val Ile Thr
150
Val Gln Lys Thr Val Thr Ala Ile Lys Thr
160
Ile Val Ile Leu Asp Ser Lys Val Asp Tyr
170
Arg Gly Tyr Gln Ser Met Asp Asn Phe Ile
180
Lys Lys Asn Thr Pro Gln Gly Phe Lys Gly
190
Ser Ser Phe Lys Thr Val Glu Val Asn Arg
200
Lys Glu Gln Val Ala Leu Ile Met Asn Ser
210
Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
220
Gln Leu Thr His Glu Asn Ala Val Thr Arg
230
Phe Ser His Ala Arg Asp Pro Ile Tyr Gly
240
Asn Gln Val Ser Pro Gly Thr Ala Ile Leu
250
Thr Val Val Pro Phe His His Gly Phe Gly
260
Met Phe Thr Thr Leu Gly Tyr Leu Thr Cys

270
Gly Phe Arg Ile Val Met Leu Thr Lys Phe
280
Asp Glu Glu Thr Phe Leu Lys Thr Leu Gln
290
Asp Tyr Lys Cys Ser Ser Val Ile Leu Val
300
Pro Thr Leu Phe Ala Ile Leu Asn Arg Ser
310
Glu Leu Leu Asp Lys Tyr Asp Leu Ser Asn
320
Leu Val Glu Ile Ala Ser Gly Gly Ala Pro
330
Leu Ser Lys Glu Ile Gly Glu Ala Val Ala
340
Arg Arg Phe Asn Leu Pro Gly Val Arg Gln
350
Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala
360
Ile Ile Ile Thr Pro Glu Gly Asp Asp Lys
370
Pro Gly Ala Ser Gly Lys Val Val Pro Leu
380
Phe Lys Ala Lys Val Ile Asp Leu Asp Thr
390
Lys Lys Thr Leu Gly Pro Asn Arg Arg Gly
400
Glu Val Cys Val Lys Gly Pro Met Leu Met
410
Lys Gly Tyr Val Asp Asn Pro Glu Ala Thr
420
Arg Glu Ile Ile Asp Glu Glu Gly Trp Leu
430
His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu

440
Glu Lys His Phe Phe Ile Val Asp Arg Leu
450
Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
460
Val Pro Pro Ala Glu Leu Glu Ser val Leu
470
Leu Gln His Pro Asn Ile Phe Asp Ala Gly
480
Val Ala Gly Val Pro Asp Pro Ile Ala Gly
490
Glu Leu Pro Gly Ala Val Val Val Leu Glu
500
Lys Gly Lys Ser Met Thr Glu Lys Glu Val
510
Met Asp Tyr Val Ala Ser Gln Val Ser Asn
520
Ala Lys Arg Leu Arg Gly Gly Val Arg Phe
530
Val Asp Glu Val Pro Lys Gly Leu Thr Gly
540
Lys Ile Asp Gly Lys Ala Ile Arg Glu Ile

Leu Lys Lys Pro Val Ala Lys Met

3. Gène de luciférase selon la revendication 1 ou 2 qui est représenté par la séquence nucléotidique présentée ci-dessous:

30
ATG GAA AAC ATG GAG AAC GAT GAA AAT ATT
60
GTG TAT GGT CCT GAA CCA TTT TAC CCT ATT
90
GAA GAG GGA TCT GCT GGA GCA CAA TTG CGC

120
AAG TAT ATG GAT CGA TAT GCA AAA CTT GGA
150
GCA ATT GCT TTT ACT AAC GCA CTT ACC GGT
180
GTC GAT TAT ACG TAC GCC GAA TAC TTA GAA
210
AAA TCA TGC TGT CTA GGA GAG GCT TTA AAG
240
AAT TAT GGT TTG GTT GTT GAT GGA AGA ATT
270
GCG TTA TGC AGT GAA AAC TGT GAA GAA TTC
300
TTT ATT CCT GTA TTA GCC GGT TTA TTT ATA
330
GGT GTC GGT GTG GCT CCA ACT AAT GAG ATT
360
TAC ACT CTA CGT GAA TTG GTT CAC AGT TTA
390
GGC ATC TCT AAG CCA ACA ATT GTA TTT AGT
420
TCT AAA AAA GGA TTA GAT AAA GTT ATA ACT
450
GTA CAA AAA ACG GTA ACT GCT ATT AAA ACC
480
ATT GTT ATA TTG GAC AGC AAA GTG GAT TAT
510
AGA GGT TAT CAA TCC ATG GAC AAC TTT ATT
540
AAA AAA AAC ACT CCA CAA GGT TTC AAA GGA
570
TCA AGT TTT AAA ACT GTA GAA GTT AAC CGC
600
AAA GAA CAA GTT GCT CTT ATA ATG AAC TCT

630
TCG GGT TCA ACC GGT TTG CCA AAA GGT GTG
660
CAA CTT ACT CAT GAA AAT GCA GTC ACT AGA
690
TTT TCT CAC GCT AGA GAT CCA ATT TAT GGA
720
AAC CAA GTT TCA CCA GGC ACG GCT ATT TTA
750
ACT GTA GTA CCA TTC CAT CAT GGT TTT GGT
780
ATG TTT ACT ACT TTA GGC TAT CTA ACT TGT
810
GGT TTT CGT ATT GTC ATG TTA ACG AAA TTT
840
GAC GAA GAG ACT TTT TTA AAA ACA CTG CAA
870
GAT TAC AAA TGT TCA AGC GTT ATT CTT GTA
900
CCG ACT TTG TTT GCA ATT CTT AAT AGA AGT
930
GAA TTA CTC GAT AAA TAT GAT TTA TCA AAT
960
TTA GTT GAA ATT GCA TCT GGC GGA GCA CCT
990
TTA TCT AAA GAA ATT GGT GAA GCT GTT GCT
1020
AGA CGT TTT AAT TTA CCG GGT GTT CGT CAA
1050
GGC TAT GGT TTA ACA GAA ACA ACC TCT GCA
1080
ATT ATT ATC ACA CCG GAA GGC GAT GAT AAA
1110
CCA GGT GCT TCT GGC AAA GTT GTG CAA TTA

1140
TTT AAA GCA AAA GTT ATC GAT CTT GAT ACT
1170
AAA AAA ACT TTG GGC CCG AAC AGA CGT GGA
1200
GAA GTT TGT GTA AAG GGT CCT ATG CTT ATG
1230
AAA GGT TAT GTA GAT AAT CCA GAA GCA ACA
1260
AGA GAA ATC ATA GAT GAA GAA GGT TGG TTG
1290
CAC ACA GGA GAT ATT GGG TAT TAC GAT GAA
1320
GAA AAA CAT TTC TTT ATC GTG GAT CGT TTG
1350
AAG TCT TTA ATC AAA TAC AAA GGA TAT CAA
1380
GTA CCA CCT GCT GAA TTA GAA TCT GTT CTT
1410
TTG CAA CAT CCA AAT ATT TTT GAT GCC GGC
1440
GTT GCT GGC GTT CCA GAT CCT ATA GCT GGT
1470
GAG CTT CCG GGA GCT GTT GTT GTA CTT GAA
1500
AAA GGA AAA TCT ATG ACT GAA AAA GAA GTA
1530
ATG GAT TAC GTT GCT AGT CAA GTT TCA AAT
1560
GCA AAA CGT TTG CGT GGT GGT GTC CGT TTT
1590
GTG GAC GAA GTA CCT AAA GGT CTC ACT GGT
1620
AAA ATT GAC GGT AAA GCA ATT AGA GAA ATA

CTG AAG AAA CCA GTT GCT AAG ATG

4. Nouvel ADN recombiné comprenant un ADN vecteur dans lequel est inséré un gène codant la luciférase dérivée de Luciola lateralis selon les revendications 1 à 3.

5. Nouvel ADN recombiné selon la revendication 4, dans lequel ledit ADN vecteur est le plasmide pUC119.

6. Procédé de production d'une luciférase qui comprend la culture dans un milieu d'un micro-organisme appartenant au genre Eschirichia et protant un ADN recombiné selon la revendication 4 obtenu par insertion d'un gène codant la luciférase dérivée de Luciola lateralis dans un ADN vecteur et l'obtention de ladite luciférase à partir de la culture.

7. Procédé de production d'une luciférase selon la revendication 6, dans lequel ledit ADN vecteur est le plasmide pUC119.

FIG. 1

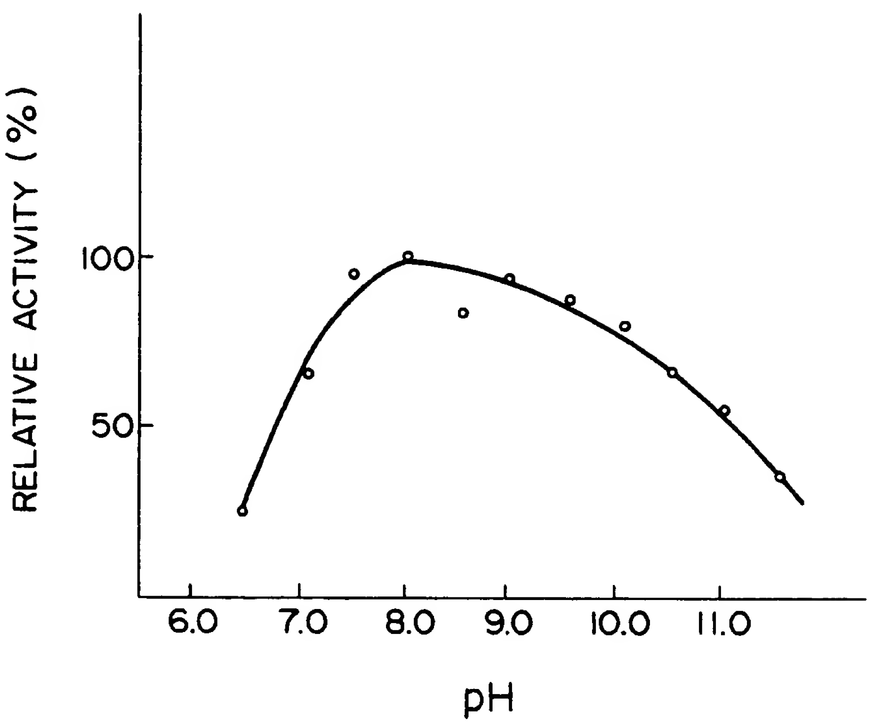


FIG. 2

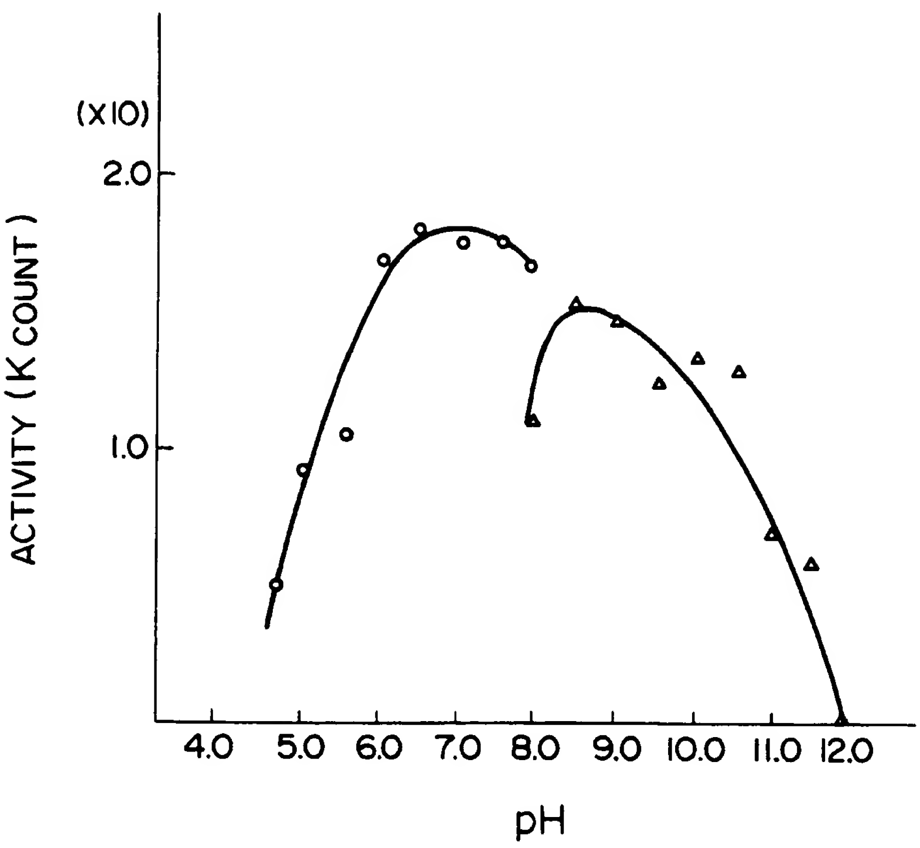


FIG. 3

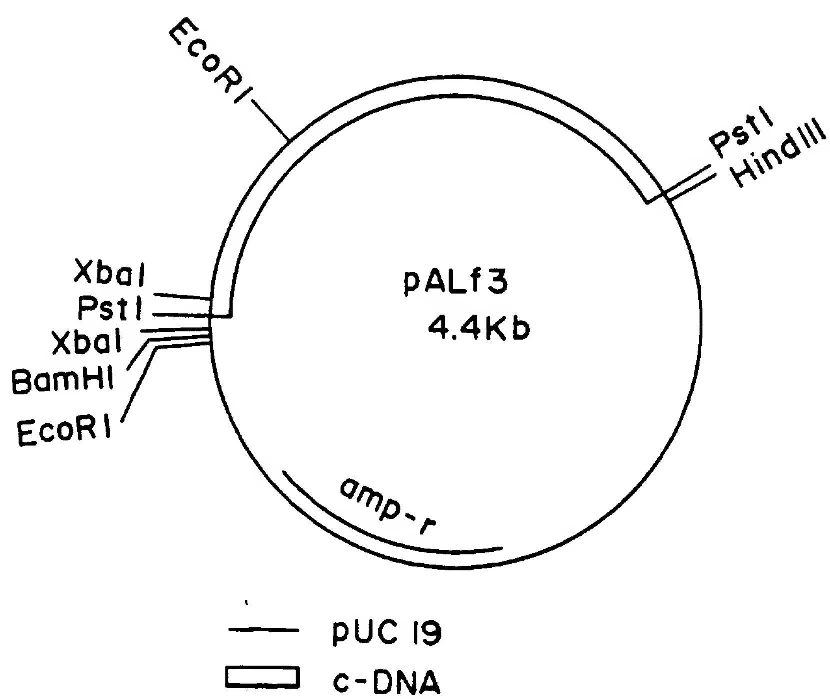

EcoRI
PstI
HindIII
XbaI
PstI
XbaI
BamHI
EcoRI
pALf3
4.4Kb
amp-r
pUC 19
c-DNA

FIG. 4

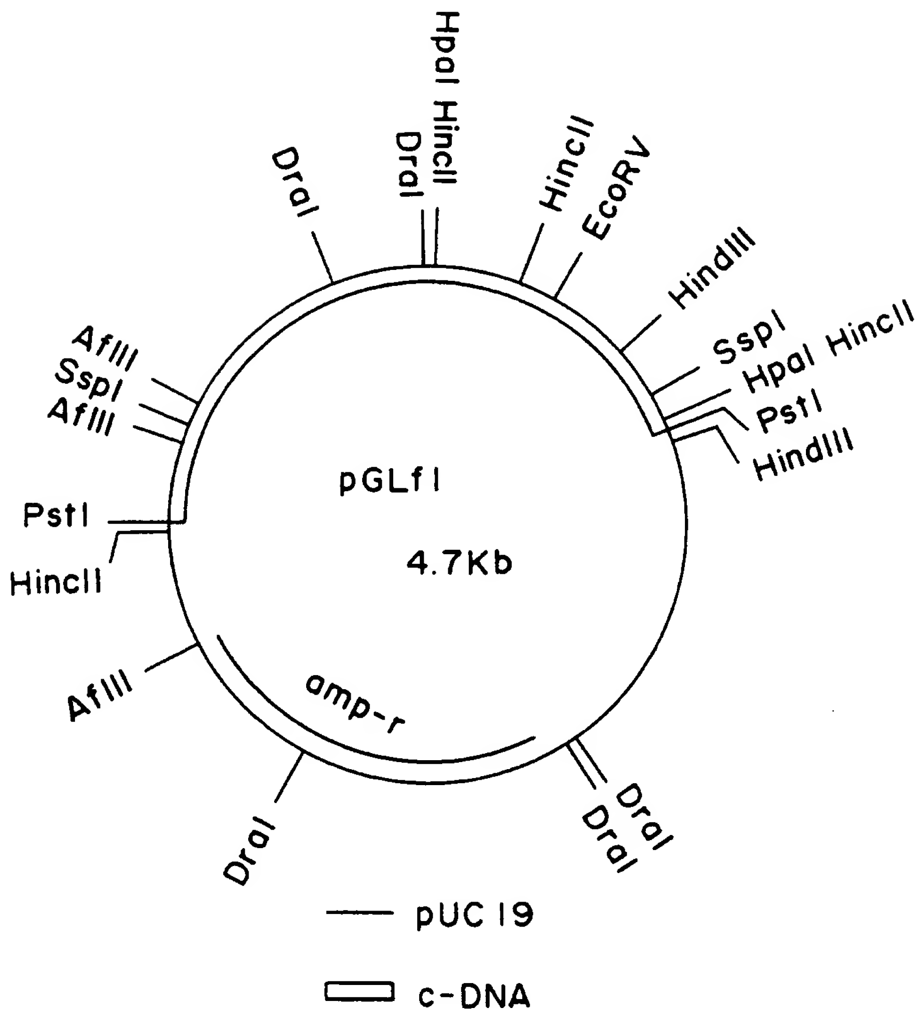

HpaI HincII
DraI
DraI
HincII
EcoRV
HindIII
SspI
HpaI HincII
PstI
HindIII
AflII
SspI
AflII
PstI
HincII
pGLf1
4.7Kb
AflII
amp-r
DraI
DraI
DraI
pUC 19
c-DNA

FIG. 5

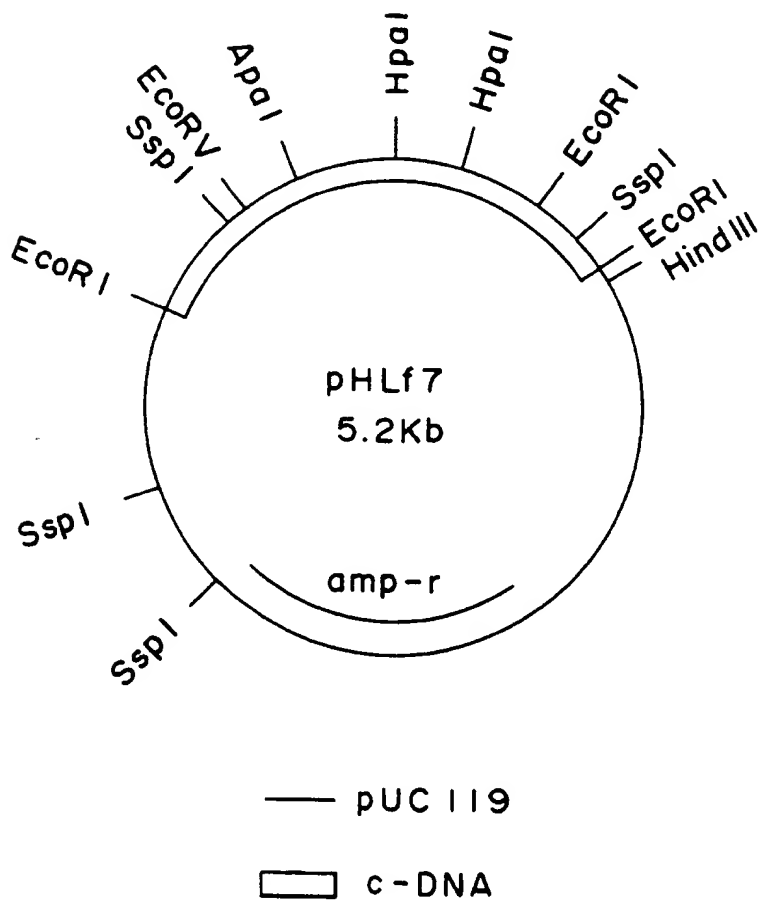

HpaI
HpaI
ApaI
EcoRV
SspI
EcoRI
SspI
EcoRI
HindIII
EcoRI
pHLf7
5.2Kb
SspI
SspI
amp-r
pUC 119
c-DNA

FIG. 6A

ATG	GAA	AAC	ATG	GAG	AAC	GAT	GAA	AAT	ATT	30
GTG	TAT	GGT	CCT	GAA	CCA	TTT	TAC	CCT	ATT	60
GAA	GAG	GGA	TCT	GCT	GGA	GCA	CAA	TTG	CGC	90
AAG	TAT	ATG	GAT	CGA	TAT	GCA	AAA	CTT	GGA	120
GCA	ATT	GCT	TTT	ACT	AAC	GCA	CTT	ACC	GGT	150
GTC	GAT	TAT	ACG	TAC	GCC	GAA	TAC	TTA	GAA	180
AAA	TCA	TGC	TGT	CTA	GGA	GAG	GCT	TTA	AAG	210
AAT	TAT	GGT	TTG	GTT	GTT	GAT	GGA	AGA	ATT	240
GCG	TTA	TGC	AGT	GAA	AAC	TGT	GAA	GAA	TTC	270
TTT	ATT	CCT	GTA	TTA	GCC	GGT	TTA	TTT	ATA	300
GGT	GTC	GGT	GTG	GCT	CCA	ACT	AAT	GAG	ATT	330
TAC	ACT	CTA	CGT	GAA	TTG	GTT	CAC	AGT	TTA	360
GGC	ATC	TCT	AAG	CCA	ACA	ATT	GTA	TTT	AGT	390
TCT	AAA	AAA	GGA	TTA	GAT	AAA	GTT	ATA	ACT	420
GTA	CAA	AAA	ACG	GTA	ACT	GCT	ATT	AAA	ACC	450
ATT	GTT	ATA	TTG	GAC	AGC	AAA	GTG	GAT	TAT	480
AGA	GGT	TAT	CAA	TCC	ATG	GAC	AAC	TTT	ATT	510
AAA	AAA	AAC	ACT	CCA	CAA	GGT	TTC	AAA	GGA	540
TCA	AGT	TTT	AAA	ACT	GTA	GAA	GTT	AAC	CGC	570

FIG. 6B

```
                                                600
AAA GAA CAA GTT GCT CTT ATA ATG AAC TCT
                                                630
TCG GGT TCA ACC GGT TTG CCA AAA GGT GTG
                                                660
CAA CTT ACT CAT GAA AAT GCA GTC ACT AGA
                                                690
TTT TCT CAC GCT AGA GAT CCA ATT TAT GGA
                                                720
AAC CAA GTT TCA CCA GGC ACG GCT ATT TTA
                                                750
ACT GTA GTA CCA TTC CAT CAT GGT TTT GGT
                                                780
ATG TTT ACT ACT TTA GGC TAT CTA ACT TGT
                                                810
GGT TTT CGT ATT GTC ATG TTA ACG AAA TTT
                                                840
GAC GAA GAG ACT TTT TTA AAA ACA CTG CAA
                                                870
GAT TAC AAA TGT TCA AGC GTT ATT CTT GTA
                                                900
CCG ACT TTG TTT GCA ATT CTT AAT AGA AGT
                                                930
GAA TTA CTC GAT AAA TAT GAT TTA TCA AAT
                                                960
TTA GTT GAA ATT GCA TCT GGC GGA GCA CCT
                                                990
TTA TCT AAA GAA ATT GGT GAA GCT GTT GCT
                                               1020
AGA CGT TTT AAT TTA CCG GGT GTT CGT CAA
                                               1050
GGC TAT GGT TTA ACA GAA ACA ACC TCT GCA
                                               1080
ATT ATT ATC ACA CCG GAA GGC GAT GAT AAA
                                               1110
CCA GGT GCT TCT GGC AAA GTT GTG CCA TTA
                                               1140
TTT AAA GCA AAA GTT ATC GAT CTT GAT ACT
```

FIG. 6C

AAA AAA ACT TTG GGC CCG AAC AGA CGT GGA 1170

GAA GTT TGT GTA AAG GGT CCT ATG CTT ATG 1200

AAA GGT TAT GTA GAT AAT CCA GAA GCA ACA 1230

AGA GAA ATC ATA GAT GAA GAA GGT TGG TTG 1260

CAC ACA GGA GAT ATT GGG TAT TAC GAT GAA 1290

GAA AAA CAT TTC TTT ATC GTG GAT CGT TTG 1320

AAG TCT TTA ATC AAA TAC AAA GGA TAT CAA 1350

GTA CCA CCT GCT GAA TTA GAA TCT GTT CTT 1380

TTG CAA CAT CCA AAT ATT TTT GAT GCC GGC 1410

GTT GCT GGC GTT CCA GAT CCT ATA GCT GGT 1440

GAG CTT CCG GGA GCT GTT GTT GTA CTT GAA 1470

AAA GGA AAA TCT ATG ACT GAA AAA GAA GTA 1500

ATG GAT TAC GTT GCT AGT CAA GTT TCA AAT 1530

GCA AAA CGT TTG CGT GGT GGT GTC CGT TTT 1560

GTG GAC GAA GTA CCT AAA GGT CTC ACT GGT 1590

AAA ATT GAC GGT AAA GCA ATT AGA GAA ATA 1620

CTG AAG AAA CCA GTT GCT AAG ATG

FIG. 7A

Met Glu Asn Met Glu Asn Asp Glu Asn Ile 10
Val Tyr Gly Pro Glu Pro Phe Tyr Pro Ile 20
Glu Glu Gly Ser Ala Gly Ala Gln Leu Arg 30
Lys Tyr Met Asp Arg Tyr Ala Lys Leu Gly 40
Ala Ile Ala Phe Thr Asn Ala Leu Thr Gly 50
Val Asp Tyr Thr Tyr Ala Glu Tyr Leu Glu 60
Lys Ser Cys Cys Leu Gly Glu Ala Leu Lys 70
Asn Tyr Gly Leu Val Val Asp Gly Arg Ile 80
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe 90
Phe Ile Pro Val Leu Ala Gly Leu Phe Ile 100
Gly Val Gly Val Ala Pro Thr Asn Glu Ile 110
Tyr Thr Leu Arg Glu Leu Val His Ser Leu 120
Gly Ile Ser Lys Pro Thr Ile Val Phe Ser 130
Ser Lys Lys Gly Leu Asp Lys Val Ile Thr 140
Val Gln Lys Thr Val Thr Ala Ile Lys Thr 150
Ile Val Ile Leu Asp Ser Lys Val Asp Tyr 160
Arg Gly Tyr Gln Ser Met Asp Asn Phe Ile 170
Lys Lys Asn Thr Pro Gln Gly Phe Lys Gly 180
Ser Ser Phe Lys Thr Val Glu Val Asn Arg 190

FIG. 7B

Lys Glu Gln Val Ala Leu Ile Met Asn Ser 200
Ser Gly Ser Thr Gly Leu Pro Lys Gly Val 210
Gln Leu Thr His Glu Asn Ala Val Thr Arg 220
Phe Ser His Ala Arg Asp Pro Ile Tyr Gly 230
Asn Gln Val Ser Pro Gly Thr Ala Ile Leu 240
Thr Val Val Pro Phe His His Gly Phe Gly 250
Met Phe Thr Thr Leu Gly Tyr Leu Thr Cys 260
Gly Phe Arg Ile Val Met Leu Thr Lys Phe 270
Asp Glu Glu Thr Phe Leu Lys Thr Leu Gln 280
Asp Tyr Lys Cys Ser Ser Val Ile Leu Val 290
Pro Thr Leu Phe Ala Ile Leu Asn Arg Ser 300
Glu Leu Leu Asp Lys Tyr Asp Leu Ser Asn 310
Leu Val Glu Ile Ala Ser Gly Gly Ala Pro 320
Leu Ser Lys Glu Ile Gly Glu Ala Val Ala 330
Arg Arg Phe Asn Leu Pro Gly Val Arg Gln 340
Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala 350
Ile Ile Ile Thr Pro Glu Gly Asp Asp Lys 360
Pro Gly Ala Ser Gly Lys Val Val Pro Leu 370
Phe Lys Ala Lys Val Ile Asp Leu Asp Thr 380

FIG. 7C

									390
Lys	Lys	Thr	Leu	Gly	Pro	Asn	Arg	Arg	Gly
									400
Glu	Val	Cys	Val	Lys	Gly	Pro	Met	Leu	Met
									410
Lys	Gly	Tyr	Val	Asp	Asn	Pro	Glu	Ala	Thr
									420
Arg	Glu	Ile	Ile	Asp	Glu	Glu	Gly	Trp	Leu
									430
His	Thr	Gly	Asp	Ile	Gly	Tyr	Tyr	Asp	Glu
									440
Glu	Lys	His	Phe	Phe	Ile	Val	Asp	Arg	Leu
									450
Lys	Ser	Leu	Ile	Lys	Tyr	Lys	Gly	Tyr	Gln
									460
Val	Pro	Pro	Ala	Glu	Leu	Glu	Ser	Val	Leu
									470
Leu	Gln	His	Pro	Asn	Ile	Phe	Asp	Ala	Gly
									480
Val	Ala	Gly	Val	Pro	Asp	Pro	Ile	Ala	Gly
									490
Glu	Leu	Pro	Gly	Ala	Val	Val	Val	Leu	Glu
									500
Lys	Gly	Lys	Ser	Met	Thr	Glu	Lys	Glu	Val
									510
Met	Asp	Tyr	Val	Ala	Ser	Gln	Val	Ser	Asn
									520
Ala	Lys	Arg	Leu	Arg	Gly	Gly	Val	Arg	Phe
									530
Val	Asp	Glu	Val	Pro	Lys	Gly	Leu	Thr	Gly
									540
Lys	Ile	Asp	Gly	Lys	Ala	Ile	Arg	Glu	Ile
Leu	Lys	Lys	Pro	Val	Ala	Lys	Met		

FIG. 8A

									10
ATG	GAA	AAC	ATG	GAG	AAC	GAT	GAA	AAT	ATT
Met	Glu	Asn	Met	Glu	Asn	Asp	Glu	Asn	Ile
									20
GTG	TAT	GGT	CCT	GAA	CCA	TTT	TAC	CCT	ATT
Val	Tyr	Gly	Pro	Glu	Pro	Phe	Tyr	Pro	Ile
									30
GAA	GAG	GGA	TCT	GCT	GGA	GCA	CAA	TTG	CGC
Glu	Glu	Gly	Ser	Ala	Gly	Ala	Gln	Leu	Arg
									40
AAG	TAT	ATG	GAT	CGA	TAT	GCA	AAA	CTT	GGA
Lys	Tyr	Met	Asp	Arg	Tyr	Ala	Lys	Leu	Gly
									50
GCA	ATT	GCT	TTT	ACT	AAC	GCA	CTT	ACC	GGT
Ala	Ile	Ala	Phe	Thr	Asn	Ala	Leu	Thr	Gly
									60
GTC	GAT	TAT	ACG	TAC	GCC	GAA	TAC	TTA	GAA
Val	Asp	Tyr	Thr	Tyr	Ala	Glu	Tyr	Leu	Glu
									70
AAA	TCA	TGC	TGT	CTA	GGA	GAG	GCT	TTA	AAG
Lys	Ser	Cys	Cys	Leu	Gly	Glu	Ala	Leu	Lys
									80
AAT	TAT	GGT	TTG	GTT	GTT	GAT	GGA	AGA	ATT
Asn	Tyr	Gly	Leu	Val	Val	Asp	Gly	Arg	Ile
									90
GCG	TTA	TGC	AGT	GAA	AAC	TGT	GAA	GAA	TTC
Ala	Leu	Cys	Ser	Glu	Asn	Cys	Glu	Glu	Phe
									100
TTT	ATT	CCT	GTA	TTA	GCC	GGT	TTA	TTT	ATA
Phe	Ile	Pro	Val	Leu	Ala	Gly	Leu	Phe	Ile
									110
GGT	GTC	GGT	GTG	GCT	CCA	ACT	AAT	GAG	ATT
Gly	Val	Gly	Val	Ala	Pro	Thr	Asn	Glu	Ile
									120
TAC	ACT	CTA	CGT	GAA	TTG	GTT	CAC	AGT	TTA
Tyr	Thr	Leu	Arg	Glu	Leu	Val	His	Ser	Leu

FIG. 8B

									130
GGC	ATC	TCT	AAG	CCA	ACA	ATT	GTA	TTT	AGT
Gly	Ile	Ser	Lys	Pro	Thr	Ile	Val	Phe	Ser
									140
TCT	AAA	AAA	GGA	TTA	GAT	AAA	GTT	ATA	ACT
Ser	Lys	Lys	Gly	Leu	Asp	Lys	Val	Ile	Thr
									150
GTA	CAA	AAA	ACG	GTA	ACT	GCT	ATT	AAA	ACC
Val	Gln	Lys	Thr	Val	Thr	Ala	Ile	Lys	Thr
									160
ATT	GTT	ATA	TTG	GAC	AGC	AAA	GTG	GAT	TAT
Ile	Val	Ile	Leu	Asp	Ser	Lys	Val	Asp	Tyr
									170
AGA	GGT	TAT	CAA	TCC	ATG	GAC	AAC	TTT	ATT
Arg	Gly	Tyr	Gln	Ser	Met	Asp	Asn	Phe	Ile
									180
AAA	AAA	AAC	ACT	CCA	CAA	GGT	TTC	AAA	GGA
Lys	Lys	Asn	Thr	Pro	Gln	Gly	Phe	Lys	Gly
									190
TCA	AGT	TTT	AAA	ACT	GTA	GAA	GTT	AAC	CGC
Ser	Ser	Phe	Lys	Thr	Val	Glu	Val	Asn	Arg
									200
AAA	GAA	CAA	GTT	GCT	CTT	ATA	ATG	AAC	TCT
Lys	Glu	Gln	Val	Ala	Leu	Ile	Met	Asn	Ser
									210
TCG	GGT	TCA	ACC	GGT	TTG	CCA	AAA	GGT	GTG
Ser	Gly	Ser	Thr	Gly	Leu	Pro	Lys	Gly	Val
									220
CAA	CTT	ACT	CAT	GAA	AAT	GCA	GTC	ACT	AGA
Gln	Leu	Thr	His	Glu	Asn	Ala	Val	Thr	Arg
									230
TTT	TCT	CAC	GCT	AGA	GAT	CCA	ATT	TAT	GGA
Phe	Ser	His	Ala	Arg	Asp	Pro	Ile	Tyr	Gly
									240
AAC	CAA	GTT	TCA	CCA	GGC	ACG	GCT	ATT	TTA
Asn	Gln	Val	Ser	Pro	Gly	Thr	Ala	Ile	Leu

FIG. 8C

250
ACT GTA GTA CCA TTC CAT CAT GGT TTT GGT
Thr Val Val Pro Phe His His Gly Phe Gly

260
ATG TTT ACT ACT TTA GGC TAT CTA ACT TGT
Met Phe Thr Thr Leu Gly Tyr Leu Thr Cys

270
GGT TTT CGT ATT GTC ATG TTA ACG AAA TTT
Gly Phe Arg Ile Val Met Leu Thr Lys Phe

280
GAC GAA GAG ACT TTT TTA AAA ACA CTG CAA
Asp Glu Glu Thr Phe Leu Lys Thr Leu Gln

290
GAT TAC AAA TGT TCA AGC GTT ATT CTT GTA
Asp Tyr Lys Cys Ser Ser Val Ile Leu Val

300
CCG ACT TTG TTT GCA ATT CTT AAT AGA AGT
Pro Thr Leu Phe Ala Ile Leu Asn Arg Ser

310
GAA TTA CTC GAT AAA TAT GAT TTA TCA AAT
Glu Leu Leu Asp Lys Tyr Asp Leu Ser Asn

320
TTA GTT GAA ATT GCA TCT GGC GGA GCA CCT
Leu Val Glu Ile Ala Ser Gly Gly Ala Pro

330
TTA TCT AAA GAA ATT GGT GAA GCT GTT GCT
Leu Ser Lys Glu Ile Gly Glu Ala Val Ala

340
AGA CGT TTT AAT TTA CCG GGT GTT CGT CAA
Arg Arg Phe Asn Leu Pro Gly Val Arg Gln

350
GGC TAT GGT TTA ACA GAA ACA ACC TCT GCA
Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala

360
ATT ATT ATC ACA CCG GAA GGC GAT GAT AAA
Ile Ile Ile Thr Pro Glu Gly Asp Asp Lys

FIG. 8D

										370
CCA	GGT	GCT	TCT	GGC	AAA	GTT	GTG	CCA	TTA	
Pro	Gly	Ala	Ser	Gly	Lys	Val	Val	Pro	Leu	
										380
TTT	AAA	GCA	AAA	GTT	ATC	GAT	CTT	GAT	ACT	
Phe	Lys	Ala	Lys	Val	Ile	Asp	Leu	Asp	Thr	
										390
AAA	AAA	ACT	TTG	GGC	CCG	AAC	AGA	CGT	GGA	
Lys	Lys	Thr	Leu	Gly	Pro	Asn	Arg	Arg	Gly	
										400
GAA	GTT	TGT	GTA	AAG	GGT	CCT	ATG	CTT	ATG	
Glu	Val	Cys	Val	Lys	Gly	Pro	Met	Leu	Met	
										410
AAA	GGT	TAT	GTA	GAT	AAT	CCA	GAA	GCA	ACA	
Lys	Gly	Tyr	Val	Asp	Asn	Pro	Glu	Ala	Thr	
										420
AGA	GAA	ATC	ATA	GAT	GAA	GAA	GGT	TGG	TTG	
Arg	Glu	Ile	Ile	Asp	Glu	Glu	Gly	Trp	Leu	
										430
CAC	ACA	GGA	GAT	ATT	GGG	TAT	TAC	GAT	GAA	
His	Thr	Gly	Asp	Ile	Gly	Tyr	Tyr	Asp	Glu	
										440
GAA	AAA	CAT	TTC	TTT	ATC	GTG	GAT	CGT	TTG	
Glu	Lys	His	Phe	Phe	Ile	Val	Asp	Arg	Leu	
										450
AAG	TCT	TTA	ATC	AAA	TAC	AAA	GGA	TAT	CAA	
Lys	Ser	Leu	Ile	Lys	Tyr	Lys	Gly	Tyr	Gln	
										460
GTA	CCA	CCT	GCT	GAA	TTA	GAA	TCT	GTT	CTT	
Val	Pro	Pro	Ala	Glu	Leu	Glu	Ser	Val	Leu	
										470
TTG	CAA	CAT	CCA	AAT	ATT	TTT	GAT	GCC	GGC	
Leu	Gln	His	Pro	Asn	Ile	Phe	Asp	Ala	Gly	
										480
GTT	GCT	GGC	GTT	CCA	GAT	CCT	ATA	GCT	GGT	
Val	Ala	Gly	Val	Pro	Asp	Pro	Ile	Ala	Gly	

FIG. 8E

									490
GAG	CTT	CCG	GGA	GCT	GTT	GTT	GTA	CTT	GAA
Glu	Leu	Pro	Gly	Ala	Val	Val	Val	Leu	Glu
									500
AAA	GGA	AAA	TCT	ATG	ACT	GAA	AAA	GAA	GTA
Lys	Gly	Lys	Ser	Met	Thr	Glu	Lys	Glu	Val
									510
ATG	GAT	TAC	GTT	GCT	AGT	CAA	GTT	TCA	AAT
Met	Asp	Tyr	Val	Ala	Ser	Gln	Val	Ser	Asn
									520
GCA	AAA	CGT	TTG	CGT	GGT	GGT	GTC	CGT	TTT
Ala	Lys	Arg	Leu	Arg	Gly	Gly	Val	Arg	Phe
									530
GTG	GAC	GAA	GTA	CCT	AAA	GGT	CTC	ACT	GGT
Val	Asp	Glu	Val	Pro	Lys	Gly	Leu	Thr	Gly
									540
AAA	ATT	GAC	GGT	AAA	GCA	ATT	AGA	GAA	ATA
Lys	Ile	Asp	Gly	Lys	Ala	Ile	Arg	Glu	Ile
CTG	AAG	AAA	CCA	GTT	GCT	AAG	ATG		
Leu	Lys	Lys	Pro	Val	Ala	Lys	Met		